Studies on dietary copper on zinc binding to ovine alpha 2-macroglobulin

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STUDIES OF DIETARY COPPER ON ZINC BINDING TO OVINE ALPHA 2-MACROGLOBULIN

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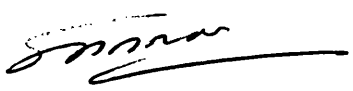

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This thesis is dedicated to my family

ABSTRACT

STUDIES OF DIETARY COPPER ON ZINC-BINDING TO OVINE ALPHA2-MACROGLOBULIN

A hypothesis was proposed that dietary copper level influences the Zn-binding to alpha 2-macroglobulin (alpha2-M) in ovine plasma and is dependent on the ratio of dietary concentrations of Cu and Zn.

Sheep plasma alpha2-M was purified and was used in raising antiserum in rabbits for use in immunoadsorbent chromatography (IAC).

Alpha2-M showed a high capacity for Zn-binding and the relationship between Zn and alpha2-M was linear, in the absence of copper.

Incubation of alpha2-M with various Cu concentrations for varying periods showed on PAGE that Zn-binding was related to Cu concentration and to the period of incubation with alpha2-M. Zn-binding was significantly ($P<0.001$) less after alpha2-M was incubated with Cu for 24 hours. This strongly indicated that Cu and Zn were probably competing for the same sites on alpha2-M.

Zn-binding to alpha2-M was studied at two levels (150 and 1500 μg Zn) of Zn incubation. In Cu-deficient sheep, plasma alpha2-M showed significantly ($P<0.001$) increased

Zn-binding. Whereas, in high-Cu diet fed sheep plasma alpha2-M showed significantly ($P<0.001$) decreased Zn-binding. The differences in Zn-binding in Cu-deficient and high-Cu sheep at both levels of Zn indicated the existence of different Zn-binding sites on alpha2-M.

During the study of Zn-binding *in vivo* to alpha2-M, the method also showed Zn-bound to the major Zn-binding protein in plasma, albumin. These studies showed a shift in Zn-binding towards alpha2-M in Cu-deficient sheep plasma, which was absent in high-Cu sheep.

The *in vitro* results were confirmed by further studies on Zn-binding in sheep fed Cu-deficient and high-Cu diets. Analysis of purified alpha2-M from the plasma of sheep fed the Cu-deficient diet showed higher Zn and no Cu. Whereas, alpha2-M from the plasma of sheep fed high-Cu diet showed presence of Cu but less Zn than Cu-deficient alpha2-M. Thus these results confirmed the observations of the *in vitro* influence of Cu and showed further evidence that Cu competes with Zn for binding sites on alpha2-M. These results also indicated two types of binding sites differing in their Zn-binding affinity.

The results from three breeds of pregnant ewes maintained indoors on a variable Cu,Zn diet in the ratio 1:4, further confirmed the influence of dietary Cu on Zn-binding to alpha2-M.

SECTION-I

REVIEW OF LITERATURE

INTRODUCTION

The essential metabolic role of dietary copper and its importance in animal nutrition has been the subject of considerable research, but inspite of this many nutritional and clinical problems remain to be solved. Similarly biochemical interactions between Cu and Zn have also been recognized but the significance and mechanisms of these interactions is unclear. It is beyond the scope of this thesis to review all the studies; hence,an attempt has been made to review the essential requirement of Cu and Zn in the nutrition of sheep and the interrelationships between these two metals. This was followed by a review of human plasma alpha2-macroglobulin (alpha2-M) a Zn-binding protein. Most of the studies were carried out in human plasma alpha2-M and not many studies are reported on ovine alpha2-M. Alpha2-M is reviewed as a Zn-binding protein and the role of zinc transport by alpha2-M is highlighted. At the end of the review the need for the proposed dietary influence of Cu on Zn-binding to sheep plasma alpha2-M is established.

ALPHA2-MACROGLOBULIN(ALPHA2-M)

This is a high molecular weight plasma glycoprotein and has been known as an inhibitor of several serine proteinases. It seems that Jacobsson (1953, 1955) was amongst the first to recognize the importance of this protein and Brown et al (1954) characterized it according to its carbohydrate content of 8.4 per cent. Schultze and colleagues first isolated alpha2-M from human serum in 1955, but for a long time its biological function remained obscure.

In animals studies on alpha2-macroglobulins have mainly concentrated on the isolation of this protein from the plasma of a variety of species: dog (Ohlsson, 1971), rabbit (Picard and Heremans, 1963; Got et al, 1965; Lebreton de Vonne and Mouray, 1968), mouse (Greene et al, 1971), pig (Jacquot-Armand and Guinard, 1967; Tsuru et al, 1975), horse (Pepper, 1968; Lavergne and Raynaud, 1970), rat (Ganrot, 1973); cow (Nagasawa et al, 1970) and hedge hog (Picard et al, 1966). None of these reports attempted to study the binding properties of alpha2-M, and somewhat surprising no reports have been found on alpha2-M in sheep with exception of the studies of allotypes of alpha2-M (Curtain, 1971). The large amount of work done, mainly in human serum, has been reviewed by Steinbuch (1971), Heimburger et al (1971), Bourrillon and Razafimahaleo (1972), Barrett and Starkey (1973), Laurell

and Jeppson (1975), Harpel (1976), Starkey and Barrett (1977) and Barrett (1981) and is summarised below.

COMPOSITION, STRUCTURE AND PROPERTIES OF HUMAN ALPHA2-MACROGLOBULIN

The amino acid composition of alpha2-M has been determined (Bourrillon and Razafimahaleo, 1972; Hamberg et al, 1973; Heimburger et al, 1964; Dunn and Spiro, 1967; Demaille et al, 1970) and a variable number of terminal NH_2 groups have been described. Schultze (1960) identified aminoterminal serine, valine, and aspartic acid, and later Razafimahaleo et al (1969) showed additional amino acids, glutamic acid, alanine and glycine.

A variety of molecular weight for human alpha2-M have been reported ranging from 650,000 by Saunders et al (1971) to 840,000 by Parisi and Vallee (1970), Schonenberger et al (1968) and Dunn and Spiro (1967). Other workers agree on 725,000 daltons (Jones et al, 1972; Barrett and Starkey, 1973 and Roberts et al, 1974). The probable explanation for these different values is differences in technique.

The structure of the alpha2-M molecule has been investigated by several investigators (Isliker, 1958; Schonenberger et al 1968; Poulik, 1960; Schultze et al, 1962; Gentou, 1968; Razafimahaleo et al, 1969; Frenoy et al,

1972; Jones et al, 1972; Harpel, 1973; Roberts et al, 1974; Barrett et al, 1979). The alpha2-M molecule is a tetramer of identical subunits linked in pairs by disulphide bonds, the two half molecules are joined by non-covalent bonds but there is no agreement as to its subunit structure (Gentou, 1968; Barrett et al, 1979).

Alpha2-M has the unique property of inhibiting proteinases specifically. Barrett and Starkey (1973) have proposed a molecular mechanism for this interaction and described three important aspects of it: (1) Many proteinases interact with alpha2-M and as they represent all four classes of these enzymes (Hartley, 1960) they differ widely in catalytic mechanism and specificity. (2) The inhibition of proteinase by alpha2-M is accompanied by binding to alpha2-M. Barrett and Starkey (1973) postulated that binding is irreversible, which is the most important aspect of the interaction with alpha2-M and that the inhibition was a secondary result of binding. (3)In the presence of alpha2-M, the reactivity of a proteinase with high-molecular-weight substrates and inhibitors typically is decreased to a much greater degree than that with low-molecular-weight compounds. This aspect of the interaction is particularly relevant to the 'trap' hypothesis of interaction of alpha2-M with proteinases.

In the 'trap' hypothesis, Barrett and Starkey (1973)

proposed that the binding of an enzyme by alpha2-M was a two stage process. The first stage consisted of proteolytic attack by the enzyme on alpha2-M molecule, which has a region (the 'bait' region) susceptible to limited proteolysis(Barrett, 1981; Van Leuven, 1982) and resulting in peptide bond cleavage. In the second stage, a conformational change occurs such that the enzyme molecule is irreversibly trapped within the alpha2-M molecule. The inhibition of enzyme activity was suggested to be the result of steric hindrance of access of substrates to the enzyme in its enclosed environment.

Evidence for conformational change was briefly reported by Morelis et al (1969) for the trypsin-rabbit alpha2-M reaction. This was extended to human and porcine alpha2-M by Barrett et al (1974) and they obtained electron micrographs which clearly demonstrated the change in shape on binding of a proteinase to alpha2-M. Barrett et al (1979) also attributed the change in electrophoretic mobility of alpha2-M after treatment with ammonium salts to conformational change.

There is evidence by Barrett et al (1979) to show that changes in electrophoretic mobility of alpha2-M could occur after treatment with ammonium salts.

Alpha2-M may dissociate into half molecules but there is confusion among the reported observations on alpha2-M

dissociation. Schonenberger et al (1968) showed that alpha2-M dissociates into half molecules in 5M urea, whereas Jones et al (1972) found that although half molecules formed in 3M urea, the dimeric subunits tended to aggregate spontaneously, unless thiol groups were alkylated. In contrast to the above reports, McConnell and Loeb (1974) were able to remove the urea without reassociation or aggregation of fragments, by dialysis and gel filtration chromatography. Alpha2-M also dissociated into half molecules after treatment with dodecyl sulphate under non reducing conditions (Harpel, 1973).

Alpha2-M may be inactivated by temperature changes. Abe and Nagai (1972) reported inactivation of alpha2-M by repeated freezing and thawing which possibly caused dissociation. However, Gentou (1968) reported that freezing and thawing caused dissociation which was reversed during 30 minutes at 20° C. Later Harpel (1973) confirmed the observations of Gentou (1968). Loss of functional activity could be caused by repeated freezing and thawing at warmer temperatures, but quick freeze in dry ice and acetone mixture and storage at -70° C preserved the enzyme binding activity of alpha2-M for at least one year (Steinbuch et al, 1968; Harpel, 1976).

Barrett et al (1979) found using gel electrophoresis that native alpha2-M has two readily distinguishable forms.

The plasma which is capable of binding, proteinases was called the electrophoretically 'slow' S-form and this could be converted to the electrophoretically 'fast' F-form after treating with either ammonium sulphate or methylammonium chloride. There is no evidence to date of the reverse change from F to S-form. They observed that freshly isolated alpha2-M ran as a single zone of low mobility in pore-limit electrophoresis and in rate electrophoresis. The observations by Barrett et al (1979) a gradual change of the S-form into the F-form with the appearance of transitional forms of intermediate mobility, after storage between -20°C and 4°C in various buffers (pH 5-6.5) may explain the 5 bands of human alpha2-M reported by Saunders et al (1971) and the doublet in human alpha2-M seen by Zais and Roberts (1977).

Isoelectric focusing of alpha2-M has been reported by several workers. Four molecular forms were reported by Frenoy and Bourrillon (1974) and Barrett et al (1979) using a pH gradient of 4-6. Ohlsson and Skude (1976) reported a change in pI of alpha2-M from 5 to 6 associated with the complexing of a proteinase but were unable to confirm this when they electrofocused an equal mixtures of inactivated S-alpha2-M and F-alpha2-M. Both forms were in the same fractions at pH 5.2.

Alpha2-M exhibits affinity of a hydrophobic type as seen

by binding to endotoxin (Yoshoko and Konno, 1970), phenyl-Sepharose, liposomes (Black and Gregoriadis (1976) and various cell membranes (Nachman and Harpel, 1976). There is no evidence to show that F or S alpha2-M differ in hydrophobic properties. However, it is not clear whether this has any part to play in the rapid clearance of F-alpha2-M from the circulation (Ohlsson, 1971), or whether there is any binding difference between the S and F-forms of alpha2-M.

Immunological studies showed a relationship between human alpha2-M and a component of animal sera (James, 1965; Butler and Brunner, 1967). In these studies, rabbit anti-human serum, adsorbed with sera in the monkey, goat, sheep, cow, donkey, zebra, dog, cat, pig and guinea pig (James, 1965); sera from sheep and rhesus monkey exhibited strong cross reaction with human alpha2-M.

Although alpha2-M was characterized in 1955 by Schultze and his coworkers, it was not until 1970 that Parisi and Vallee were able to characterize human alpha2-M as a zinc metalloprotein. They showed that alpha2-M accounts for 30 to 40% of the total zinc content of serum or plasma, with the remaining 60-70% associated with albumin (Vikbladh, 1951; Vessel and Bearn, 1957). Zinc is firmly bound to alpha2-M in plasma (Adham et al, 1977). The implications of this distribution of zinc could have significance in several disease conditions (Vallee, 1959; Parisi and

Vallee, 1969).

A number of factors may influence the concentration of circulating alpha2-M. In children, there is 2.5 times as much alpha2-M as in adults (180 mg/100 ml) (Ganrot and Shersten, 1967). There is no agreement on the concentration levels of alpha2-M in serum of adults, but the range in males is from 150-370 mg/100 ml serum and in females from 265-460 mg/100 ml serum (James et al, 1966a). The suggestion that females have higher levels of alpha2-M than males (Ganrot and Schersten, 1967; James et al, 1966; Housley, 1968); was not confirmed by Adham et al (1968). Increases in alpha2-M concentration have been observed in pregnancy (Horne et al, 1970; Ganrot and Bjerre, 1967). Ganrot and Bjerre (1967) reported that in early pregnancy alpha2-M concentration in serum reached a steady state of about 20% higher than before pregnancy and was maintained at that level for some days after parturition.

Elevated alpha2-M values have also been recorded in the following disease states: systemic lupus erythromatosus (Norberg et al, 1970); hypogammaglobulinemia (Norberg et al, 1970; James et al, 1966a), ataxia telangiectasia , atopic dermatitis mangolism (James et al, 1966a); nephrotic syndrome (Housely, 1968; Peterkofsky et al, 1956; Hartman et al, 1958; Schultze and Schwick, 1959;

Steines and Mehl, 1966); pulmonary disorders, diabetes mellitus and agammaglobulinemia (James et al, 1966a) and in patients with liver disease (Housely, 1968; Damus and Wallace, 1975). Virji et al (1985) demonstrated the effective use of alpha2-M as a diagnostic tool in differentiating bacterial and aseptic meningitis. They observed the mean cerebrospinal fluid (CSF) concentration of alpha2-M was 15 times higher in the bacterial meningitis group.

PURIFICATION OF HUMAN ALPHA 2-MACROGLOBULIN

Alpha2-M is precipitated by ammonium sulphate in the range of 1.6 - 1.92 M, at pH 6 (Bourrillon and Razafimahaleo, 1972). However, Mehl et al (1964) pointed out the risk of inactivation of alpha2-M by ammonia during fractionation with ammonium sulphate and this was later re-emphasized by the observation of Heimburger et al (1971) that the inactivation occurs at pH 8 but not at pH 5. However, Hamberg et al (1973) and Tsuru et al (1975) successfully used ammonium sulphate for the fractionation of human alpha2-M. Harpel (1973) and Giroux (1975) used polyethylene glycol and showed complete precipitation at 4-12% and 10% respectively. Sucrose density gradient centrifugation of human serum proteins also yielded pure alpha2-M (Song and Adham, 1979). Preparative isoelectric focusing in the range of pH 4-6 has been used in the purification of alpha2-M by Green et al (1971), Berne et al (1972), Hamberg et al (1973) and

Frenoy and Bourrillon (1974).

Lipoproteins may be removed by precipitation with 0.4% Rivanol (6, 9-diamino-2-ethoxyacridine lactate) (Barrett and Starkey, 1973; Song et al, 1975), by precipitation with dextran sulphate, Ca^{2+} or Mn^{2+} (Frenoy et al, 1972; Barrett and Starkey, 1973), or by ultracentrifugation (Havel et al, 1955) effectively.

THe high molecular weight of alpha2-M proved advantageous in purification step, because of its total exclusion from Sephadex G-200 (Pharmacia) and near exclusion from Sepharose 6B (Pharmacia). Starkey and Barrett (1977) obtained best results from Ultrogel ACA22 (LKB Instruments Ltd.).

Immunoglobulins have been removed by anion exchange chromatography (Steinbuch et al, 1965; Jones et al, 1972; Harpel, 1973) and preparative electrophoresis (Bloth et al, 1968; Harpel, 1973; and Hamberg et al, 1973).

In general, there is always a risk of contamination by haptoglobin during purification and this can be solved by using haptoglobin type 1-1 plasma or by adsorption on to haemoglobin covalently linked to Sepharose 4B (Starkey and Barrett, 1973).

The discovery by Porath et al (1975) that chelate forming

ligands for metal ions (Cu, Zn, Cd, Hg, Co and Ni) could be attached to agarose derivatives has provided the basis for the purification of alpha2-M. Zinc chelate affinity chromatography has been used by several workers (Lebreton, 1977; Kurecki et al, 1979; Sottrup-Jensen, 1980; Sinosich, 1984).

The methods described so far used large quantities of plasma or serum (200-500 ml) gave yields of less than 40 per cent and were not convenient for individual samples. Virca et al (1978) described a simple method combining gel filtration and affinity chromatography on Cibacron Blue Sepharose, in which recovery of alpha2-M was 75 percent. This was further modified by Bridges et al (1982) giving a recovery of 98 per cent pure alpha2-M from as little as 5 ml plasma. This method is applicable to all plasma samples regardless of haptoglobin type. Immunoadsorbent chromatography method (McEntire, 1978) utilizing rabbit antihuman alpha2-M conjugated Sepharose 4B also offered a rapid, simple and inexpensive method for purifying alpha2-M from as little as 2 ml of plasma or serum.

The above review of alpha2-M has shown in particular it relates entirely to human alpha2-M and very few studies are reported in ovine species.

ASSAY AND DETECTION OF ALPHA2-M

Trypsin binding activity was originally used for the assay of alpha2-M (Haverback et al, 1962); Mehl et al, 1964; Ganrot, 1966). However, because of the two readily distiguishable forms of alpha2-M in gel electrophoresis (Barrett et al, 1979) determination of total alpha2-M is best quantified immunologically. The total concentration of alpha2-M in solution has been determined by radial immunodiffusion (Barrett and Starkey, 1973) and 'rocket' immunoelectrophoresis (Weeke, 1973); using a reference serum of known alpha2-M concentration.

Barrett (1981) reported a better procedure which depends upon the inhibition of thermolysin activity on dyed hide powder. Hide thermolysin is rather selective, being inhibited by few other proteins. The substrate used, hide powder, is of such a large particle size that the steric inhibition by alpha2-M was complete (Barrett et al, 1979; Rinderknecht et al, 1975).

ESSENTIAL REQUIREMENT FOR COPPER

McHargue (1926) first suggested that copper was an essential requirement in the diet of rats. But conclusive evidence of the biological requirement for copper, was offered by Hart et al (1928), working with anaemic milk-fed rats. They reported that the anaemia was not corrected by iron supplementation alone, or by a liver extract alone, but by feeding iron and liver extract together they observed a marked increase in the haemoglobin and packed cell volume, within two weeks. A bluish tinge of the ashed preparation was a clue to its copper content and later this important discovery was confirmed and extended to other species.

Most mammals consume diets containing copper in excess of minimum daily requirements (Schroeder et al, 1966). The first indication of copper deficiency occuring naturally were reported in live stock in the 1930s (Neal et al, 1931; Sjollema, 1933 & 1938). Bennetts and Chapman (1937) also reported that enzootic ataxia (swayback) was a manifestation of inadequate intake of copper.

Anaemia was shown as a common expression of copper deficiency (Beck, 1941a & b). The copper deficiency was characterised by a slow reduction in body copper stores, including a fall in the copper concentration in plasma until the concentration was below the normal level

required to maintain a normal rate of haematopoiesis. Beck (1941) and Marston et al (1948) showed that levels below 0.12 μg Cu/ml plasma limit blood formation in sheep.

A number of disorders have been associated with copper deficiency or with response to copper therapy. They include depressed growth, bone disorders, depigmentation of hair and wool, abnormal wool growth, neonatal ataxia, impaired reproductive performance, heart failure, cardiovascular defects and gasterointestinal disturbances. The degree of disorder due to copper deficiency varies with the species and the stage of maturity of the animal (Underwood, 1971).

Copper is the prosthetic element of several enzymes which are essential for mammalian life. Any deficiency of the metal may lead to clinical abnormalities (Sternlieb, 1980; Committee on Medical and Biologic Effects of Environmental Pollutants, 1977). Schultze (1939) showed greatly decreased cytochrome oxidase activity of liver and the ventricles of copper deficient rats. Later studies by Labbe and Fischer (1984a & b) in rats observed reduced activity of serum ceruloplasmin, liver and heart superoxide dismutase and heart cytochrome c oxidase. Similarly, Saylor and Leach (1980) reported a lower ceruloplasmin activity and lower direct reacting copper concentrations in the plasma of lambs fed on copper

deficient diet when compared with control lambs during a period of 120 days.

ESSENTIAL ROLE OF ZINC

The first indication that zinc has a function in higher animals was published by Birckner (1919). Attempts to experimentally demonstrate such a function using semipurified diets met with limited success (Bertrand and Benson, 1922; Hubbell and Mendel, 1927; McHargue, 1926), until Todd, Elvehjem and Hart (1934) produced the first indisputable evidence for zinc as a dietary essential for the rat. 20 years later, Tucker and Salmon (1955) made the important discovery that zinc cures and prevents parakeratosis in pigs. Various investigations reported growth retardation and testicular atrophy as a result of experimentally induced zinc deficiency in animal species such as rats (Follis et al, 1941; Todd et al, 1934), calves (Miller and Miller, 1960), lambs (Ott et al , 1964; Mills et al, 1967; Pierson, 1966; Underwood and Somers, 1969) and dogs (Robertson and Burns, 1963).

Zinc is present in the plasma and in erythrocytes, leucocytes and platelets. About two thirds of the plasma zinc is loosely associated with albumin and the remainder is more firmly bound to globulins (Prasad and Oberleas, 1970 & Prasad, 1976). A small percentage of zinc has been shown to be ultrafilterable. In vitro studies indicated that zinc could combine with transferrin (Sorgenor et al,

1949; Boyett and Sullivan, 1970; Scott and Bradwell, 1983). The whole of the zinc in erythrocytes is bound to carbonic anhydrase (Hove et al, 1940).

Mills et al (1967) showed a rapid fall in plasma zinc concentration in lambs and calves after 36 hours which continued to decrease to less than half the control values within a week when fed diets contained less than 1 mg Zn/kg of diet. They concluded from their studies that ruminants had a capacity of converting tissue stored zinc to the mobilized form in the plasma, thus explaining the rapid fall in plasma zinc when dietary zinc deficiency was practised.

Abundant evidence has accrued to show that zinc is involved in protein synthesis, nucleic, amino acid and carbohydrate metabolism, bone formation and many other essential metabolic processes in most mammals (Li, 1966; Matrone, 1970; Miller, 1970; Mills, 1980; Nelsen et al, 1970; Parisi and Vallee, 1970; Somers and Underwood, 1969; Underwood, 1962). Consequently as would be expected many pathological effects resulting from dietary zinc deficiency and dietary zinc excess have been observed in ruminants (Miller, 1970; Mills et al, 1969; Prasad, 1966; Underwood, 1962; Rao, 1977, Parry and Rao, 1975, 1977, 1981; Parry et al, 1984). Unfortunately few of these conditions have been fully understood with regard to their biochemical mechanisms; thus nutritional control or

an avoidance of an imbalance is still not practical.

DIETARY REQUIREMENTS OF COPPER AND ZINC

The recommended copper requirements for sheep is 5 mg Cu/kg dry diet and the toxic level is 8-25 mg Cu/kg, while the recommended requirement for zinc is 35-50 mg Zn/kg diet and the toxic level is 1000 mg Zn/kg diet (The Nutrient Requirements of Ruminant Livestock, 1980). However, ambiguity does arise in the optimum level of dietary copper required to produce symptoms of copper toxicity in sheep and the form in which it is available is an important consideration (Todd and Thompson, 1963; Todd et al, 1962).

DIETARY RATIOS OF COPPER AND ZINC

Much has been published on the essential role of copper and zinc as individual nutrients in mammalian metabolism; whilst considering these elements as a nutritional ratio has received less attention (Bremner and Marshall, 1974a & b; Klevay, 1977; Taper et al, 1980; Parry and Al-Mukhtar, 1980; Parry et al, 1984).

An example of how the ratio of Cu to Zn can become important can be illustrated by the work of Klevay (1975c). His work has developed a hypothesis based on the ratio of Cu to Zn (Klevay, 1973, 1974a-c, 1975a-c, 1976a-c, Klevay and Forbush, 1976; Klevay et al, 1975,

1976). This hypothesis states that a "metabolic imbalance in regard to zinc and copper is a major factor" in the etiology of ischaemic heart disease. This metabolic imbalance is "either a relative or an absolute deficiency of copper characterized by a high ratio of zinc to copper". Klevay's studies in rats produced evidence that the effect on cholesterol metabolism was produced by an alteration in the ratio of zinc to copper and not by other dietary component closely associated with copper or zinc. In a similar experiment, Looney et al (1977) found that dietary high zinc and low copper produced greater hypercholesterolemia in rats than a diet low in copper.

Again, work by Parry and Rao (1981) showed in rabbits that the ratio of Zn to Cu was important in the level of rabbit plasma cholesterol; they related the concentration of plasma cholesterol to the ratio rather than to an individual element. It is now becoming far more important to consider these two elements together especially in association with a molecule such as alpha2-M.

DIETARY EXCESS OF COPPER AND ZINC, COMPETITION BETWEEN COPPER AND ZINC AND ANTAGONISM BETWEEN COPPER AND ZINC

There are numerous examples of metabolic disorders resulting from inadequate supply and excess of both zinc and copper (Marston and Lee, 1948; Marston et al, 1948; Lafanetre et al, 1935; Todd and Thompson, 1963; Todd et al, 1962; Todd, 1969; Ishmael et al, 1971 and 1972;

Saylor et al, 1980; Labbe and Fischer, 1984a & b). However, much research needs to be done on the elucidation of the biochemistry of these disorders.

There are fewer reports on high dietary zinc than studies on dietary zinc deficiency because the clinical symptoms of deficiency have been more easily observed and defined.

The antagonism between Zn and Cu was studied in rats by Sutton and Nelson (1937) and Smith and Larson (1946) who showed that high dietary zinc (between 5000 - 10000 mg Zn/kg of diet) produced anaemia as a result of induced copper deficiency (Van Reen, 1966). Smith and Larson (1946) further showed that copper supplementation reduces anaemia in rats. Thus confirming the importance of Cu for haemoglobin formation (Hart et al, 1928).

In a series of experiments Van Reen and Pearson (1953) and Van Reen (1953) showed that a dietary level of 5000 to 7000 mg Zn/kg of diet administered to rats as zinc carbonate resulted in a marked reduction in liver catalase and cytochrome oxidase activities. However, when dietary copper was supplemented with the high zinc diets, an increase in liver catalase and cytochrome oxidase activities occured until they returned to normal values. In these later studies, while copper supplementation influenced the activities of liver enzymes, it had no effect in correcting the growth inhibition produced by

the high dietary zinc levels. Thus, this is in evidence where the antagonism effects between Zn and Cu worked on biochemical mechanisms in opposite directions.

Zinc has been shown to be a dietary essential to maintain normal growth and performance (Ott et al, 1964). However, they reported certain differences from those of rats when high dietary zinc was fed to lambs and cattle. They observed subnormal liver copper levels and the anaemia was much less pronounced than rats and the iron concentration of the liver increased in lambs and cattle (Ott et al, 1966a & b). Contrary to the reports by Sadasivan (1951a, b & 1952) observing defective development and demineralisation of bone in zinc-toxic rats, Ott et al (1966a & b) produced evidence showing depressed feed consumption, reduced gain, decreased feed efficiency and increased mineral consumption as a result of high zinc diets. Prolonged consumption of high levels of zinc can however cause death. Furthermore,the lambs refused to eat the basal diet supplemented with 4000 mg Zn/kg diet, this suggested that the high concentration of zinc in the diet could effect the palatability.

Sheep are very sensitive to excess copper. The biochemical reasoning for this is not clear but it is becoming obvious that zinc and a binding protein such as alpha2-M might have been promising parameters to measure in previous publications. Excessive copper accumulation

appears to be one of the hazards when sheep are maintained indoors for prolonged periods (Todd, 1969) or grazing pastures with high copper content. Contamination of feeds with copper compounds from horticultural sources has been the cause of excess copper intake. Losses of sheep from dietary excess of copper, with haemolysis, icterus and haemoglobinuria as characteristic signs, have been reported from many parts of the world (Lafenetre et al, 1935; Gracey and Todd, 1960). In some cases the excess copper has come from the ingestion of herbage in orchards and vineyards previously sprayed with copper compounds (Lafenetre et al, 1935).

Rats appeared to be extremely tolerant of high copper intakes. Normal growth and health was maintained on diets containing 500 mg Cu/kg of diet, which was 100 times the normal concentration of the diet, despite a fourteen fould increase in liver copper (Boyden et al, 1938).

It has been demonstrated that excessive copper concentrations are toxic to sheep (National Research Council, 1977; Mahoney et al, 1955; Bremner, 1974; Ishmael et al, 1971; Gopinath et al, 1974; Todd and Thompson, 1963; Todd et al, 1962), to ruminants (Todd, 1969; National Research Council, 1977; Bremner, 1974; Shand and Lewis, 1957; Weiss and Baur, 1968). The copper accumulates primarily in liver, where the storage capacity varies with the species (Sternlieb, 1980). In

sheep, the phase of copper accumulation or the prehaemolytic phase may take place over a period of 6 to 10 weeks or longer. During this time liver copper levels increase (on a dry weight basis) gradually from a normal range of 6 to 279 mg Cu/kg weight of liver,to a range of 1000 to 3000 mg Cu/kg weight of liver (McCosker, 1968). However, the whole blood copper concentration remains within normal limits of 70 to 120 µg Cu/100 ml blood for the first half of the prehaemolytic period. During the second half of the prehaemolytic period concentration of copper attains twice their normal value and then suddenly increases by 8 to 10 times the normal value coinciding with the haemolytic crisis. During the same period a rise in serum glutamic oxalacetic transaminase (SGOT) values have been observed (Thompson and Todd, 1974).

An early clinical symptom of copper toxicity in sheep during the prehaemolytic period is gradual loss in weight. Luke and Marquering (1972) showed that 100 µg Cu increase per kg liver results in an 8.5 g loss in daily weight gain.

It has become apparent in recent years that when sheep are kept indoors for extended periods, chronic copper poisoning is one of the possible hazards. Evidence to this effect was produced by Bracewell (1958), who showed 24 out of 720 ewes housed for periods of upto 16 months, died of haemolytic jaundice. A mineral supplement

containing 43 mg Cu/kg of diet was fed for the first 5 months, but at this point first cases of copper poisoning occured and the supplement was withdrawn thereafter. Despite this, further cases appeared during the following 11 months. This was also observed by Senior (1959). However, Hemmingway and MacPherson (1967) pointed out that the possibility of copper poisoning is not great under normal feeding system, with no supplementary copper added to the diet of lambs. They stressed the existence of great variability between individual animals in their ability to accumulate copper, but stress could precipitate the toxic syndrome.

Studies by van der Berg et al (1983) showed significant increases in copper accumulation in the livers of four breeds of sheep fed on high copper diet (35 mg Cu/kg of diet). Though the copper level of diet given to the sheep was the same, copper accumulation in the four breeds varied.

Cases of chronic copper poisoning occurred in sheep where excessive copper intakes in the feed could not be demonstrated, which led to the investigation of other factors involved. There is evidence to show a relationship between feed protein intake and copper accumulation in the liver. McCall et al (1961) reported that symptoms of toxicity, after feeding 750 mg Cu/kg diet, decreased after raising the concentration of

protein in the diet. McCall and Davis (1961) observed no significant increase in liver copper in rats fed 1000 mg Cu/kg diet when protein intake was increased by 25%; with only 10% protein intake there was a highly significant increase in liver copper accumulation. Similarly, MacPherson and Hemingway (1965) observed no accumulation of copper in livers of sheep fed on low copper intakes and an increased crude protein content, however, at high copper intakes (1 g copper sulphate/sheep/day) in the diet with high crude protein content did apparently have an effect and decreased the incidence of copper accumulation in the animals. This latter evidence indicates that the higher protein intake buffers the higher intake of Cu in some way. It is regretable that previous workers did not measure the blood proteins such as alpha2-M.

Smith and Larson (1946) first recognized a biological antagonism between copper and zinc, when they used dietary supplementation with copper to alleviate certain symptoms of zinc toxicity in rats. Later studies of Grant-Frost and Underwood (1958) observed the signs of copper deficiency in rats fed on diet containing 5000 mg Zn/kg of diet.

It is well recognized that copper metabolism in ewes and lambs is affected by increasing dietary zinc concentrations (Bremner et al, 1976; Campbell and Mills,

1979; Parry and Al-Mukhtar, 1980; Saylor et al, 1980). Bremner et al (1976) examined the possibility of dealing with copper poisoning in intensive sheep rearing by increasing their dietary zinc intake. Sheep were protected substantially by increasing the dietary zinc concentration to 420 mg Zn/kg of diet. This supplementation prevented the onset of haemolytic crisis and also decreased the amount of liver damage which normally occurs with Cu toxicity. Further evidence by Parry et al (1984) confirmed the protective effect of feeding zinc upto 352 mg Zn/kg diet. Although the biochemical explanation for this has not been worked out, it would seem reasonable to assume that Zn could have been involved in stimulating the biosynthesis of metallothionein which in turn could have bound excess Cu ions, thus rendering them unavailable for liver metabolism. Occurence of metallothionein in the liver of ruminants (Bremner and Marshall, 1974b) is dependent on zinc status. The detoxification and storage of copper have been linked with metallothionein by Bremner and Marshall (1974b).

Contrary to this evidence and views that zinc increase in the diet can decrease liver copper studies by Saylor and Leach (1980) and Reynolds (1978) showed that supplementation of the sheep diet with 500 mg Zn/kg of diet did not decrease the copper concentration of the liver. However, lambs in experiments by Bremner et al,

(1976) were 12 weeks of age whilst those used by Saylor and Leach (1980) included newborn lambs up to 60 days old. Hence, Saylor and Leach (1980) suggested that the age at which zinc supplementation took affect was more important than the duration of feeding a zinc supplemented diet.

Parry et al (1984) showed in three breeds of pregnant sheep, kept indoors that a ratio of 1:4 between dietary Cu and Zn could reduce the effect of toxic levels of copper. They used commercially available diet, with copper varying from 20 mg to 82 mg Cu/kg and zinc from 80 to 352 mg Zn/kg, for maintaining dietary ratios of 1:4 for Cu:Zn. In their binding studies on plasma protein using Dorset sheep it was shown that plasma protein bound more copper during the first 21 days but this was not apparent after a total of 105 days. These studies supported earlier results by Bremner et al (1976) in showing the significance of dietary zinc supplementation in dealing with copper toxicity.

ABSORPTION OF ZINC

A number of studies have shown that zinc metabolism is under the control of a homeostatic mechanism (Miller, 1969; Evans , 1973). Some investigators suggested a role for low molecular weight proteins, possibly such as metallothionein making a contribution to the absorption process (Starcher, 1969; Van Campen and Kowalski, 1971; Suso and Edwards, 1971). However, there is no unifying concept regarding zinc absorption and transport.

Hahn and Evans (1973) identified a low molecular weight Zn-binding ligand in the intestinal lumen and mucosa of rats. Later, Evans et al (1975) showed that a similar ligand is present in dog and rat pancreatic secretions. The Zn-binding ligands have not been characterised, in any of these species and there is certainly a scarcity of studies on these processes in sheep.

Evans et al (1975) proposed the following sequence for zinc absorption based on their observations in rats: 1. a Zn-binding ligand is secreted in pancreas and released into the intestinal lumen, 2. in the lumen zinc binds to the ligand, 3.complexed with ligand, zinc is transported through the intestinal microvillus and into the epithelial cell, 4. in the epithelial cell Zn is transported to binding sites on the baso lateral plasma membrane, and 5. metal free albumin interacts with the

plasma membrane and removes Zn from the receptor sites. The quantity of metal free albumin available at the basolateral plasma membrane determines the amount of Zn removed from the intestinal epithelial cell and thus regulates the quantity of Zn that enters the body.

Although 30-40% of serum Zn is associated with alpha2-M (Parisi and Vallee, 1970), this protein was suggested to play a minor role in Zn absorption. This was based on the observations of Parisi and Vallee (1970) indicating that alpha2-M does not take up or exchange readily with Zn isotope.

ABSORPTION OF COPPER

Studies of Evans (1979) showed that intestinal cytosol Cu binds to metallothionein. When the quantity of Cu that enters the intestinal cell does not exceed the capacity of the mucosal transport mechanism, probably occupies binding sites on metallothionein and Cu is released from metallothionein as the Cu is transported across the basement membrane into the lamina propria. However, when the quantity of incoming copper exceeds the capacity of the intestinal transport mechanism, the pre-existing binding sites on metallothionein become saturated and leads to metallothionein synthesis.

Stuydies in ruminants have shown that increased molydenum content of the diet above 1 mg Mo/kg diet causes copper

deficiency. This is further enhanced by the dietary content of inorganic sources of sulphur or sulphur amino acids (Mills, 1980). The interaction between Cu,Mo and S takes place predominantly in the gut (Suttle, 1974) and suggested that it may involve the formation of an unavailable compound containing Cu and Mo which might limit the utilisation of dietary Cu by sheep.

ZINC BINDING TO ALPHA2-M AND ZINC TRANSPORT BY ALPHA2-M

Zimmerman et al (1984) suggested that zinc bound to alpha2-M may be involved in providing readily available zinc in plasma for transport towards rapid cell growth and differentiation. While accepting albumin as the principal zinc transporting protein among tissues for rapid zinc change (to gut, liver and placenta), they suggested that alpha2-M interacts with cells to transfer zinc for complex metabolic needs such as DNA synthesis in the fetus. This view supports the rapid transport requirement role of alpha2-M to meet the increased zinc requirements during pregnancy.

Evidence to show that alpha2-M may have an important transport role comes from Laemmli (1970) who showed that metalloproteins require divalent cations i.e. Zn, Co, Mn, Mg and Ca for their activity. Later, Barrett and Starkey (1973) suggested that alpha2-M serves as a means of removal of activated proteases, which have bound metal

ions, from the circulation by forming complexes. Such studies therefore indicate that alpha2-M can be involved in transport. However, despite the evidence, that alpha2-M could have an important role for metal ions in this way there are no studies published which have investigated whether bound Zn can be released and become available for example in faetal tissue where the demand for Zn is high.

There is no agreement on the zinc transporting proteins either in human plasma or ruminant plasma. In the plasma of most species approximately two thirds of the zinc present has been found to be loosely bound and one third very firmly bound to alpha2-M (Himmelhock et al, 1966; Parisi and Vallee, 1970; Giroux, 1975). In addition a small proportion of zinc 2-8% in an ultrafilterable form is bound to the amino acids:- histidine, glutamine, threonine, cystine and lysine (Prasad and Oberleas, 1970). Although Scott and Bradwell (1983) showed <u>in vitro</u> binding of zinc to albumin and 12 other proteins in human serum, there was no evidence that binding to alpha2-M was as prominant. This could be due to the methodology used.

Studies of Boyett and Sullivan (1970) showed zinc binding consistently to transferrin and alpha2-M (these two proteins were measured together as Tf-alpha2-M). The total amount bound to these two proteins in normal and cirrhotic patients was remarkably constant (11.5 $\pm$ 2.4

µg/100 ml normal serum and 11.0 ± 5.1 µg/100 ml cirrhotic serum). However, transferrin bound zinc concentration varied in normal and cirrhotic serum samples. Hence, they implied a reciprocal relationship between the amount of zinc bound to transferrin and that bound to alpha2-M. The total zinc bound to Tf-alpha2-M remained constant even though total zinc in serum zinc decreased to as low as 12 µg/100 ml. Boyett and Sullivan (1970) predicted that Tf-alpha2-M bound zinc does not change until serum zinc levels reach below 8 µg/100 ml. More experimental evidence is needed to clarify these predictions.

In vitro studies by Prasad and Oberleas (1970) showed that amino acids: histidine, glutamine, threonine, cystine and lysine competed effectively with haptoglobin for binding of zinc, a tighter affinity for binding zinc with ceruloplasmin and alpha2-M. Other proteins such as IgG, transferrin, and albumin showed no such phenomena, which indicated that ceruloplasmin and alpha2-M showed special binding property with respect to zinc in vitro.

Charlwood (1979) reported complete in vitro transfer of zinc from transferrin to albumin at equilibrium. There are no studies so far as to whether this transfer of zinc does take place in vivo and also whether alpha2-M and amino acids are involved in transfer of zinc from one to the other either in human or in ovine species.

Very interesting observations on alpha2-M were made by Pratt and Pizzo (1984), who showed correlated decreases in human plasma alpha2-M activity (trypsin binding activity) with apparent dissociation of the alpha2-M tetramer on polyacrylamide gel electrophoresis in the presence of zinc above 25 μM. They also observed no change in the activity of alpha2-M after treatment with EDTA, which supported the view that reaction with proteinase and the resulting change in conformation of alpha2-M does not involve zinc binding sites.

Pratt and Pizzo (1984) also demonstrated that alpha2-M activity decreased in the presence of 50 μM Cu or Zn.

Evaluating the biochemical methodology which has been used for studying alpha2-M especially in binding studies is an important consideration. The same workers above found that SDS polyacrylamide gel electrophoresis of alpha2-M in 400 μM $CuSO_4$ or 400 μM of $ZnCl_2$ did not disrupt the covalently bound alpha2-M subunits. All alpha2-M-trypsin complexes incubated with $CuSO_4$ or $ZnCl_2$ showed that each of the above metal ions migrated as single bands with a mobility higher than that of untreated alpha2-M (Pratt and Pizzo, 1984).

Parisi and Vallee (1970) observed no exchange of ^{65}Zn during isolation of alpha2-M, which suggests that zinc

was bound with a high stability constant, a feature of other metalloproteins (Vallee and Wacker, 1970). However, a direct role for zinc in the activity of alpha2-M is not recognized (Adham et al, 1977; Pratt and Pizzo, 1984).

Human alpha2-M has been studied by Adham et al (1977). They found evidence to show some heterogenous binding; this was attributed to two classes of binding sites. At pH 7.9 the site with strong affinity for zinc (site 1) showed an apparent stoichiometry of nearly 12 g atoms Zn/mol of alpha2-M and an apparent association constant of $3.06.10^{7}$. The second binding site (site 2) had 60 g atoms Zn/monomer and an apparent association constant (K2) of $1.32.10^{5}$. For the third site (site 3) the calculated stiochiometry amounted to 40 g atoms Zn/mol of alpha2-M and K3 was equal to $7.33.10^{5}$. However, they indicated that the third binding site was an artefact. Binding constant estimated for the first of these sites by Pratt and Pizzo (1984) was $8 . 10^{-7}$ M and zinc binding site number of 20. Pratt and Pizzo (1984) reported the existence of low affinity binding sites as suggested by Adham et al (1977), although the upper limit for site number and the binding affinity were not accurately estimated due to the problem of protein aggregation and precipitation. They suggested that these numbers should be best considered as limiting values for a class of high-affinity zinc binding sites on human alpha2-M.

Later, Gettins and Cunningham (1986) investigated the metal binding properties of human alpha2-M and detected a unique pair of high affinity zinc sites in the alpha2-M tetramer that differ markedly from other weaker sites.They also obtained data with manganese binding to apo-alpha2-M which was very similar to that of zinc and concluded that manganese had similar relative affinities for the two types of site as with zinc.

Though it is recognized that trypsin binding activity of alpha2-M does not depend on the presence of zinc in alpha2-M (Adham et al, 1977; Parisi and Vallee, 1970; Pratt and Pizzo, 1984), however, Adham et al (1977) showed that at 20 g atoms Zn/mol, 25% of trypsin binding activity was lost and when the concentration of zinc was increased to 70 g-atoms Zn/mol alpha2-M, there was disappearance of all trypsin binding activity. The activity was not restored after removal of excess zinc from alpha2-M by dialysis, suggesting that the occupation of the second binding site of alpha2-M by zinc initiated irreversible conformational changes in the molecule interfering with enzyme binding activity.

Pratt and Pizzo (1984) observed a reduction in reassociation of the subunits in the presence of zinc (ranging from 0 to 1 mM) on polyacrylamide gel electrophoresis, after the reaction of alpha2-M with trypsin.

There is strong evidence to claim that human alpha2-M has a plasma Zn transport role. The studies by Parisi and Vallee (1970) showed that alpha2-M has a concentration range of 320-770 μg Zn/g of alpha2-M, this corresponds to 3.3-7.9 g-atoms Zn/mol of alpha2-M (molecular weight of alpha2-M:- approximately 840,000 - Schonenberger et al, 1968). They indicated a role of transport for alpha2-M on the basis of variations in zinc concentration in several disorders and due to 30-40% zinc in alpha2-M. Pratt and Pizzo also support the role in zinc transport,in view of the absence of any effect on alpha2-M structure and function at physiological zinc concentration. Alpha2-M has the capacity to bind 20 mol zinc/mol at physiological concentration, but this does not mean saturation of one class of 20 binding sites because the limit of zinc binding in serum can be upto 40%. However, the actual zinc bound to alpha2-M may be less than total binding capacity. Pratt and Pizzo also state that the reversible binding capacity of alpha2-M is consistent with the existence of many zinc binding sites.

According to Pratt and Pizzo (1984), though the structural and functional implications of zinc binding to alpha2-M at high concentrations of zinc is not physiologically relevant,but it is useful to consider the problem in this way to try and understand the protein-metal interactions,particularly with regard to

subunit association.

Song and Adham (1979) showed that approximately 80-100 μg Zn/g of protein and the molar ratio between zinc and alpha2-M as approximately 1.This ratio was also maintained in hypo and hyperzincemic sera obtained from patients with multiple myeloma and lymphocytic lymphoma.

The stability of alpha2-M bound Zn concentration in healthy human adults was reported by Foote and Delves (1984). They observed 2.4 ± 0.6 μmol Zn/l in human alpha2-M. These observations were in close agreement with Giroux et al (1976) who showed a mean Zn concentration of 2.5 ± 0.5 μmol/l. However, higher Zn concentration of 3.3 ± 0.1 μmol/l was reported by Song and Adham (1979) in alpha2-M which was obtained after sucrose density centrifugation. Foote and Delves (1984) also reported a poor correlation between the concentration of alpha2-M and bound Zn due to a fourfould variation in the binding ratio between the two respectively (0.5 to 2.2). They suggested that alpha2-M bound Zn is subject to metabolic control. However,they were unable to offer any explanation to this obscure behaviour or the importance of it.

Little has been mentioned in this review on zinc bound to albumin; it is important to consider this. Foote and Delves (1984) further reported that the variations in

human serum Zn concentrations were entirely due to variation in albumin Zn. However, further investigations are needed to provide evidence and studies on Zn-binding to alpha2-M might help in clearer understanding of the nature of Zn variation in serum.

The above review of literature has shown the areas of work carried out on human alpha2-M, essential requirement of copper, zinc in deficiency and excess, also the interrelationships between copper and zinc and the mutual antagonism between the two metals. Despite this, there are hardly any reports of studies showing the influence of one element on the binding of the other. From the review of literature it appears that mutual antagonism between copper and zinc could be used in preventing copper poisoning in sheep (Bremner et al, 1976). One of the ways in which zinc affected the toxicity of copper was by promoting the formation of relatively non-toxic forms of copper in the liver such as metallothionein. However, it is not clear whether concentration of one of these two metals could influence the binding of the other to plasma proteins and interfere with transport. It is beyond the scope of this thesis to investigate the influence on all proteins. Hence, this thesis confined the investigation of the influence of copper on Zn-binding to alpha2-M, and evidence is presented to show that it is a Zn-binding protein with a role in the transport of zinc.

AIM OF THE INVESTIGATION

The main aim of this work was to attempt a quantitative assessment of Zn-binding to alpha2-M and how this is influenced by dietary copper. The hypothesis to be tested proposes that dietary copper influences Zn-binding to alpha2-M in ovine plasma and is dependent on the ratio of dietary concentrations between Cu and Zn.

The individual aims of this work are therefore summarized below and will be a guide to the general direction of the experimental design.

a. Purification of alpha2-M from sheep plasma. This is an essential first step before it can be used to test whether ovine alpha2-M is different in character and zinc binding properties from human alpha2-M as mentioned in this thesis.

b. It is then necessary to test whether there is a binding relationship between plasma alpha2-M and Zn in sheep plasma which in turn is sensitive to various parameters summarized as follows:

i. influence of dietary copper, firstly copper deficient on Zn-binding to alpha2-M, with possible competiton between the two elements for binding sites;

ii.secondly,the possible influence of high dietary copper on Zn-binding to alpha2-M with a similar possibility of competition between the two elements as in (i) above.

c.Finally it is necessary to study whether Zn-binding to alpha2-M is different in different breeds of sheep and whether dietary copper can influence this Zn-binding.

SECTION - II

METHODS AND EXPERIMENTAL DESIGN

1.(a). GENERAL EXPERIMENTAL DESIGN AND TREATMENTS

(i). Clun Forest wethered male lambs were used to obtain blood samples from sheep fed copper deficient diet (<1.0 mg Cu/kg and 50 mg Zn/kg diet) and high-Cu diet (250 mg Cu/kg and 200 mg Zn/kg diet) fed sheep. Male lambs of 12 weeks old were used in both the above experiments.

(ii). Samples of blood were also obtained from three other experiments carried out at the Department of Clinical Veterinary Medicine, Cambridge. In these experiments three breeds of pregnant sheep (Dorset, Clun and Finn) were used. These sheep were maintained indoors on a variable Cu diet (20 to 82 mg Cu/kg diet supplemented with 80 to 352 mg Zn/kg diet respectively with a ratio Cu:Zn of 1:4.

(iii). Animal house and pens

At Bristol Polytechnic, sheep were housed in individual pens throughout the experiments; the pens were made of stainless steel bars covered with plastic sheet so that sheep could not lick them. The floors of the pens were plastic trays and plastic mats were provided to serve as bedding. The pens were washed and cleaned three times a week. Temperature of the animal house was maintained at 20°C ±1.

(iv). The sheep at the Department of Clinical Veterinary Medicine, Cambridge were accommodated and managed in a polypen (thick polythene sheeting draped over and fixed to a steel frame). Three breeds of sheep, Dorset, Clun and Finn were separated in three respective groups of 10 pregnant ewes in each. Immediately before housing these ewes were grazing a paddock with a previous history of copper toxicity causing deaths amongst each of the three breeds.

(b).DIETARY TREATMENTS

In the experiments conducted at Bristol Polytechnic and at Cambridge three types of diet were used:-high-Cu diet and varying copper-zinc diets were supplied by Dalgety Spillers, Clifton, Bristol. Cu and Zn concentrations in these diets were analysed prior to the start of each experiment.

Copper deficient diets were mixed at University of Bristol, Veterinary School, Langford, using the ingredients supplied by Dalgety Spillers. The diet was based on the composition used at the Rowett Research Institute, Aberdeen, Scotland.

Percentage composition of the copper deficient basal diet

Ingredients	% of diet
Chopped straw	30
Dry skimmed milk(DSM)	20
Maize starch	20.5
Sugar	12.5
Glucose	6.25
Arachis oil	4.00
Urea	3.00
Major minerals	
$KHCO_3$	1.024
NaCl	0.391
$MgSO_4.7H_2O$	0.912
$CaHPO_4.2H_2O$	0.971
Trace elements	
$FeSO_4.7H_2O$	0.0199
$MnSO_4.4H_2O$	0.0162
$CoCl_2.6H_2O$	0.0004
KIO_3	0.0002
$ZnSO_4.7H_2O$	0.022
Fat soluble vitamins	
A (1000 i.u./kg); D (360 i.u./kg) and E (10 i.u./kg)	0.006

B group vitamins 0.036

(B group vitamins-biotin separated from others; naphthoquinone, pyridoxine, folic acid, thiamin HCl, riboflavin, DL-calcium pantothenate (45%), vitamin B12, ascorbic acid and inositol.)

(c). COPPER AND ZINC ANALYSIS

(i).Diet

Copper and zinc analysis were made on a Varian Techtron AA6 atomic absorption spectrophotometer.

A steel heating block was used for the digestion of diet samples. The steel block could accommodate 10 boiling tubes and was of the same size as the hot plate. The steel block was placed on the hot plate for digestion as mentioned below.

2 g of powdered diet was added to each of the boiling tubes except one and then 10 ml of concentrated Analar nitric acid (Analar) was added, followed by three drops of octan-1-ol and two drops of perchloric acid. A one inch diameter glass marble was placed on top of each tube, to reduce evaporative loss. Thermal contact with the block was aided by few drops of liquid paraffin between the block and glass tube. A tenth tube with 2 ml liquid paraffin and a thermometer in it was used to monitor the temperature. The block with the tubes was

placed on the hot plate in a fume cupboard and was warmed gently until frothing was observed. When the frothing ceased the temperature was increased to 105°C. The samples were kept at this temperature for 48 hours and when they were completely dissolved, the solutions were diluted to a known volume and the zinc and copper content were measured by atomic absorption spectrophotometer.

(ii).Preparation of plasma samples

10 to 20 ml of blood from the animals jugular vein was collected into tubes containing lithium heparin. Plasma was separated by centrifuging at 3000 rpm for 15 minutes and subsequently stored in a freezer at -20° C.

When needed the plasma samples were diluted 1 in 5 with deionised water for measuring copper and zinc.

(iii).Chromatography fractions

Fractions from chromatographic separation were directly used for measuring copper and zinc.

(d).REAGENTS

(i).Stock copper-1000 µg Cu/ml

1 g of copper metal strip (Analar) was dissolved in a minimum volume of 1:1 nitric acid (Analar) and diluted to 1 litre with deionised water to give 1000 µg Cu/ml and stored in a polythene bottle.

Calibration standards

Suitable dilutions of the stock copper solution were made with deionised water, usually to give 10 μg Cu/ml solution first and then 0.05, 0.1, 0.2, 0.5 and 1.0 μg Cu/ml standards.

(ii).Stock zinc-1000 μg Zn/ml

1 g of zinc metal, granulated (Analar) dissolved in 40 ml 1:1 hydrochloric acid (Analar) and diluted to 1 litre with deionised water to give 1000 μg Zn/ml and stored in a polythene bottle.

Calibration standards

Suitable dilution of the stock zinc solution was made with deionised water, usually 10 μg Zn/ml. From this diluted stock 0.1, 0.2, 0.5, 1.0 and 2.0 μg Zn/ml standards were made.

(e).CONTAMINATION BY EXOGENOUS COPPER AND ZINC

Precautions were taken in all experiments to prevent copper and zinc contamination from external sources. All glassware was first cleaned by immersion in 35% W/V nitric acid, washed in distilled water and then rinsed with deionised water. All chemicals used were of Analar grade and all solutions were made up in deionised water and stored in polythene containers.

(f).RADIOACTIVITY COUNT

A Scaler Ratemeter SR7, Nuclear Enterprises Ltd. was used for radioactivity determination of ^{65}Zn from fractions collected from mini columns used in the binding studies. The counting time was always for one minute.

A Rackbeta II(LKB Liquid Scintillation Counter) was used for ^{65}Zn count of polyacrylamide gel slices from both rod gel and vertical gel electrophoresis. In both cases 5 mm sections of gels were placed in scintillation vials and 5 ml of liquid scintillator (Unisolve E, Koch Light Ltd.) was dispensed into each vial with an automatic dispenser. Precautions were taken by using liquid scintillator only as blank and also ^{65}Zn with liquid scintillator to monitor ^{65}Zn count for comparison. The vials were left in the rack for 6 hours to allow the gel slices to dissolve completely, after which time each vial was shaken thoroughly and then counted. This procedure gave consistently reliable counts and on recounting showed reproducibility (5%).

2. PURIFICATION OF ALPHA2-M FROM SHEEP PLASMA

There are a number of methods available, as mentioned in the review of literature, for the purification of alpha2-M from plasma. No previously described method has been used specifically to purify alpha2-M from sheep plasma. However, due to strong immunological cross reaction between sheep and human alpha2-M (James, 1965), previously described methods for human plasma purification were adapted for use in this study to purify sheep alpha2-M.

Alpha2-M is easily inactivated either by reaction with proteinases or by the procedures adopted in protein purification, hence, the risk was reduced working with plasma rather than serum. Since complex and long procedures tend to inactivate alpha2-M, it was necessary to compromise between obtaining alpha2-M free from all contaminants and in retaining the active alpha2-M.

Two published methods of preparation were prefered:

(a). A large scale purification method (Song et al, 1975) using precipitation with polyethylene glycol, centrifugation and gel filtration ,

(b). Immunoadsorbent chromatography (IAC) (McEntire, 1978) using specific anti-serum (anti-human alpha2-M)

coupled to CNBr-Sepharose 4B and eluting alpha2-M with pH 2.5 acetate buffer.

The first method offered the possibility of sufficient yield of alpha2-M for both preliminary studies and material for raising anti-sheep alpha2-M antibodies in rabbits. The latter were needed for the second method, immunoadsorbent chromatography (McEntire, 1978). These two methods are convenient for use with both large and small samples of plasma. The immunoadsorbent chromatography method was extremely convenient for routine use and was economical.

The methods are described below in detail.

(a).LARGE SCALE PURIFICATION OF ALPHA2-M FROM SHEEP PLASMA(Song et al, 1975)

Materials required

Polyethylene glycol 300 (PEG-300), Rivanol (2-ethoxy-6,9-diaminoacridine lactate) (Sigma Chemical Co., Poole, Dorset). Sephadex G-200 (Pharmacia, MIlton Keynes). Fresh sheep blood was obtained from an abattoir in polythene container with heparin in it.

Preparation of alpha2-M

(i).Polyethylene glycol precipitation

All preparations were carried out at 4° C. To 300 ml of fresh sheep plasma, 120 g of PEG-300 was added with stirring. The mixture was allowed to stand for 1 hour at

4° C and then centrifuged for 2 hours at 3000 g. The precipitate was discarded. Another 120 g of PEG-300 was added to the supernatant. The mixture was stirred, allowed to stand for an hour and centrifuged at 3000 g for 2 hours. The precipitate was dissolved in 100 ml saline and treated as below.

(ii).Removal of beta-lipoprotein and fibrinogen by Rivanol precipitation

30 ml of 0.5% Rivanol in distilled water was added to the crude alpha2-M solution in saline mixture was stirred for 10 minutes and centrifuged at 3000 g for 30 minutes. An additional 20 mls of 0.5% Rivanol was added to the supernatant. After standing for a further hour, the mixture was centrifuged for 1 hour at 3000 g. The pellet obtained was dissolved in 3% NaCl (50 ml) and clarified by centrifugation. Finally, this supernatant was passed through a Sephadex G-200 column (5 x 80 cms.) which had been equilibrated with 5 mM tris-HCl buffer, pH 7.4 containing 0.9% NaCl and 0.02% sodium azide. The flow rate of the column was 24 ml/hr and the absorbance was monitored at 280nm. A total of 100 fractions (each of 10 ml) were collected.

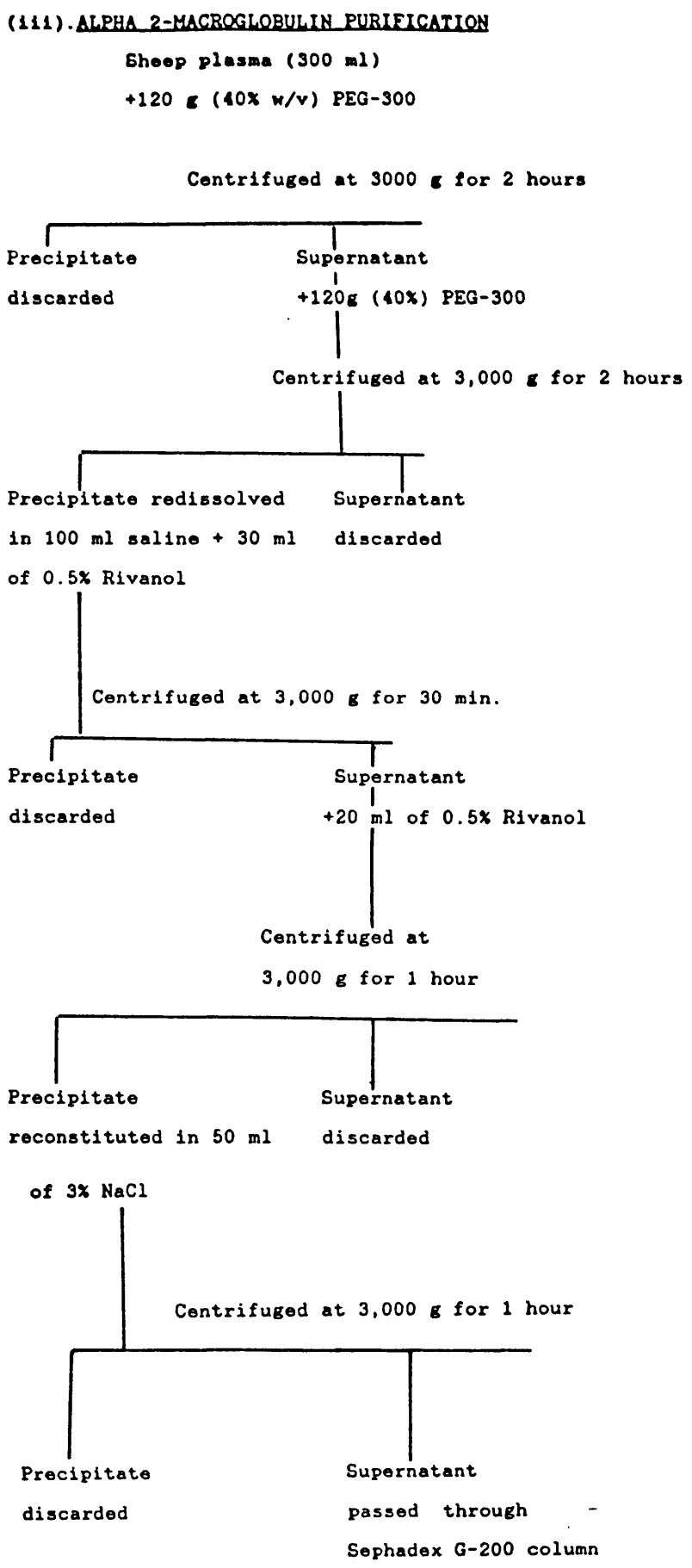
Those fractions which contained protein were tested for:

a. alpha2-M;
b. zinc concentration;
c. copper concentration.

Three distinct protein peaks will be seen in Fig.7 in Section-III. Peak I was in the region of the void volume and thus high molecular weight protein material. Prior to the chromatography of alpha2-M, the void volume of the column was determined passing 10 ml of Blue Dextran 2000 (Pharmacia) (2 mg/ml) and molecular weight markers (details in Section-II, 13) were applied to determine the molecular weight of Peak I.

Human plasma alpha2-M was also purified following the same procedure (Fig.9, Section-III).

(iii). ALPHA 2-MACROGLOBULIN PURIFICATION



Flow chart of purification of sheep plasma alpha2-M.

A typical elution pattern from this G-200 column is shown in Fig.7 (Section-III).

(b).IMMUNOADSORBENT CHROMATOGRAPHY (McEntire, 1978)

(i).Materials required

Antihuman alpha2-M raised in rabbits from Behring Diagnostic Reagents, Hoechst Pharmaceuticals, Hounslow; anti-sheep alpha2-M was raised in rabbits for use in this procedure (details in Section-II, 5); Sepharose 4B (CNBr activated) from Pharmacia (Great Britain) Ltd., Milton Keynes; 0.1 M borate buffer, pH 8.0 in 0.5 M NaCl; 1 M ethanolamine, pH 8.0; 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl and 0.1 M acetate buffer, pH 2.5.

(ii).Preparation of anti-alpha2-M immunoadsorbent column

Cyanogen bromide-activated Sepharose 4B (CNBr-Sepharose 4B) was prepared according to the manufacturers instructions as follows.

2.0 g of freeze-dried powder was swollen for 15 minutes in 1 mM HCl and washed on a sintered glass filter (porosity G3) with the same solution. A total of approximately 200 ml per gram dry gel was added in several aliquots, the supernatant removed between successive additions. 1 g of freeze-dried powder has given approximately a gel volume of 3.5 ml. The use of HCl preserved the activity of the reactive groups which hydrolyse at high pH. The gel was then washed thoroughly with coupling buffer (0.1 M borate buffer, pH 8.0, in 0.5 M NaCl) and 4 ml of anti-human alpha2-M or anti-sheep

alpha2-M (previously dialysed against coupling buffer, overnight) was added to the Sepharose and tumble mixed slowly for 2 hours at room temperature on a rotary mixer. After washing with coupling buffer, a number of residual active groups will still remain on the gel. These groups were blocked by adding an excess of a small primary amine, 1.0 M ethanolamine, pH 8.0 to the Sepharose-antibody mixture. The mixing was continued for two hours. At the end of this period, the coupled Sepharose was decanted into a small Whatman column (1.0 x 12.0 cms.) and washed thoroughly with alternating aliquots (50 ml) of coupling buffer and 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl.

Normally for the purification of alpha2-M from experimental sheep plasma, whole procedure was scaled up 5 times.

(iii).Purification of alpha2-M from sheep plasma on IAC

Sheep plasma (20 mls) was dialysed overnight against coupling buffer (pH 8.0) and then run into the prepared Sepharose column (approximately half way). Dialysed plasma was incubated for 30 minutes at room temperature. After which time the column was washed with coupling buffer, usually 250 ml, until the 280 nm absorbance of the effluent reached less than 0.01 absorbance. The bound alpha2-M was eluted with 0.1 M sodium acetate buffer, pH 2.5, containing 0.5 M NaCl. Fractions of 5.0 ml were collected. The elution profile was monitored by using a Uvicord S (LKB Instruments Ltd.) at 280 nm. After this elution the column was washed thoroughly with the elution buffer and re-equilibrated with coupling buffer before applying a further sample. Re-equilibration was always started after the elution buffer absorbance dropped to less than 0.01 A 280.

Typical profiles of this immunoadsorbent chromatography using both anti-human alpha2-M and anti-sheep alpha2-M are presented later in Figs.13 and 14 (Section-III). Fractions were pooled and concentrated.

(c).CONCENTRATION OF PROTEIN FRACTIONS

The protein fractions from both large scale purification and immunoadsorbent chromatography were pooled and concentrated against 40% solution of carbowax

(Polyethylene glycol 6000, Sigma) at 4° C.

(d).CONFIRMATION THAT THE PROTEIN IS ALPHA2-M

It was necessary to identify the protein in each of the fractions in Peak I from the large scale purification method. Once it was confirmed that they contained alpha2-M the fractions were pooled.

In immunoadsorbent chromatography all the protein fractions obtained after the sodium acetate buffer wash pooled.

(e).ALPHA2-M ANALYSIS

(i). Double immunodiffusion analysis

(ii). Immunoelectrophoresis

(iii). Polyacrylamide gel electrophoresis (PAGE).

Materials required

Anti-human alpha2-M raised in rabbits was obtained from Behring Diagnostic Reagents, Hoechst Pharmaceuticals, Hounslow; anti-whole sheep serum was obtained from Bristol Polytechnic; and agarose, Coomassie Brilliant Blue R-250 were obtained from Sigma.

(i).Double immunodiffusion analysis(Ouchterlony)

This method allows several antigens or antibodies to be compared directly around a single well of antigen or antibody. Antibody (antihuman alpha2-M) was placed in the

central well and the antigens (diluted protein fractions or undiluted protein fractions) were placed in the equidistant peripheral wells, containing either the same volume as antiserum or less. Antigen and antibody diffuse towards each other and a sharp line of precipitation occurs where the two are in optimal concentration. Multiple lines of precipitation appear if the antigen contains several molecular species.

The method was as follows:
A solution of 1.5% agarose in barbitone buffer, pH 8.6, I = 0.1 was prepared and the bottle was left in a boiling water bath continuously (when in use).

Glass slides (26 x 76 mm) were precoated with two drops of agarose and 3 ml of liquid agarose (1.5%) was poured onto each slide and allowed to set. After the gel was set, holes were punched as shown in Fig.1 with a gel puncher and antiserum was placed in the central well and either the fractions from column or concentrated sample were placed in the outer wells. The diffusion was allowed to take place in a moist chamber at room temperature for 24 hours. After which time, precipitation lines appeared which were visualized by staining as mentioned below.

Staining of agarose gels

The slide was placed on the bench and covered with a filter paper (smooth side down) soaked with distilled

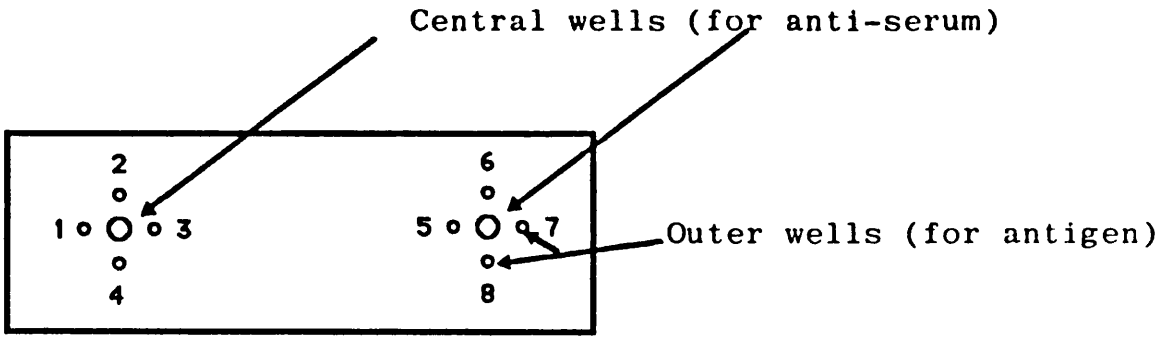


Fig.1 Glass slide showing the position of central well for anti-serum and outer wells for antigen (test solution).

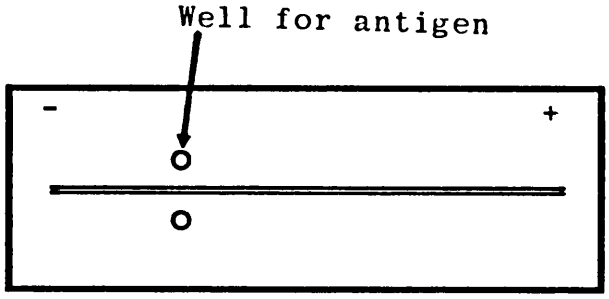


Fig.2 Immunoelectrophoresis slide showing two wells on either side of central (cut portion of gel) lines indicating the position of trough before electrophoresis.

water. A thick wad of paper tissue was placed on top of the filter paper which was covered with a thick glass plate. On this a weight (usually a winchester bottle filled with water) was placed for 20 minutes.Then the weight, wad of tissue paper and filter paper were carefully dismantled after making the filter paper wet, to avoid splitting the gel. A petri dish was half filled with distilled water and the slide was immersed and left for 20 minutes. This procedure of pressing with tissue paper and washing was repeated four times. After which time a petri dish was half filled with saline (0.9% NaCl) and left immersed for 20 minutes. Once again pressing and washing was repeated with saline instead of distilled water. Then the slide was dried completely with a hair dryer untill it was transparent and the slide was immersed in Coomasie Blue stain (1 g Coomasie Brilliant Blue R-250, 90 ml ethanol, 20 ml acetic acid and 90 ml distilled water) and stained for 5 minutes. The staining solution was used several times. After this staining the gels were rinsed with water and destained for about 10 minutes using destaining solution containing ethanol, acetic acid and water in the ratio,9:2:9.

When glass plates (84 x 94 mm) were used, 12 ml of liquid agarose (1.5%) was poured onto glass plate. The procedure used was essentially similar to slides except gel punchers (LKB Instruments Ltd.) used were different.

(ii).Immunoelectrophoresis

Principle:This technique was carried out in two steps. The antigen solution was placed in a well in the agarose gel and separated electrophoretically as shown in Fig.2.

After completion of electrophoresis, a trough was cut out of the agarose gel parallel to the direction of migration (Fig.3). The trough was then filled with the appropriate antiserum (antihuman alpha2-M or antisheep alpha2-M or antiwhole sheep serum). The antigens and antiserum diffused and formed precipitates. This was purely a qualitative technique.

Immunoelectrophoresis method for slides

A microscope slide was precoated with two drops of 1.5% agarose in barbitone buffer, pH 8.6, I = 0.05 and 3 ml of agarose was poured onto glass slide (26 x 76 mm)(a levelling table was used).

The gel was allowed to set and a trough was cut using a Shandon gel cutter. Two holes were cut 27 mm from one end of the slide and 5 mm from the trough as shown in Fig.2.

Electrophoresis

5 µl of the antigen for investigation was added to each hole. This was marked with a crystal of bromophenol blue.

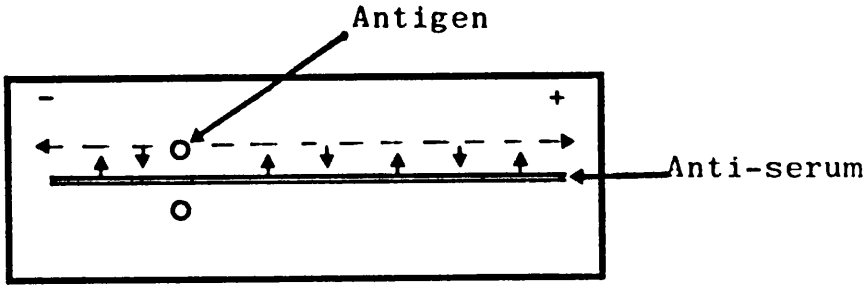


Fig.3 Immunoelectrophoresis slide with central trough filled with anti-serum after electrophoresis. Arrows indicating diffusion of anti-serum and antigen.

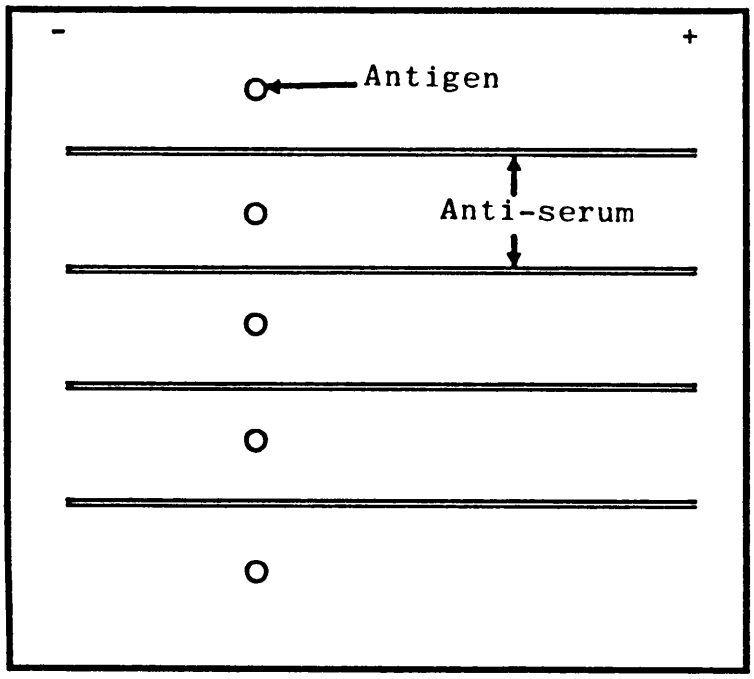


Fig.4 Glass plate for immunoelectrophoresis, showing the position of wells punched with LKB gel puncher and the troughs for anti-serum.

This dye indicated the approximate migration of the antigen. As this dye binds to albumin it was more useful when serum was used to monitor the migration of proteins. However, it was necessary to have a trial run in the presence of dye to estimate the correct time needed for the sample applied. In general 1 hour was sufficient for electrophoresis to achieve good separation.

The slide was placed across the bridge in electrophoresis tank (Shandon Southern) and separated the proteins using a potential difference of 50 volts/slide.

Diffusion

After electrophoresis the gel was carefully removed from the trough and 50 µl of the appropriate antiserum was added to the trough. Then the slide was placed in a moist diffusion chamber at room temperature for at least 24 hours. The gel was stained and destained to visualise the precipitation lines as described under double diffusion technique.

Immunoelectrophoresis on glass plates (84 x 94 x 1 mm) was performed when more than two samples were tested under the same conditions as described below.

Immunoelectrophoresis on glass plates (84 x 94 x 1 mm)- Grabar and Williams (1953)

Principle: The principle was same for microscope slide or

the glass plates as described above.

Procedure: The procedure followed was as described in the LKB Application Note 249.

In order to keep the distance between antibody and antigen and to facilitate application of more than two samples the template was used to punch up to five holes. The troughs were cut with scalpel before the sample was applied (Fig.4). However, the gel was not removed before electrophoresis. Electrophoresis was carried at 200 volts for 45 to 60 minutes depending on the number of plates used at a time.

After electrophoresis the gel was removed from the troughs using the projection on the handle of the scalpel (LKB). The troughs were then filled with 50 to 100 µl antibodies (anti-human alpha2-M or anti-sheep alpha2-M or anti-whole sheep serum or anti-IgG) as necessary. The plate was placed in a humidity chamber at room temperature and allowed to diffuse for at least 15 hours. The pressing, drying and staining procedures were the same as those described above for slides. However, it was necessary to wash the plate for a longer time (6 times) because of the high concentration of antiserum around the troughs.

(iii). Polyacrylamide gel electrophoresis (PAGE)

The high resolution achieved by PAGE is due to separation on the basis of charge and size. The method of Hendrick and Smith (1968) emphasised the importance of maintaining a constant bis/acrylamide monomer ratio, since small changes in this ratio dramatically change the crosslinking and thus the seiving characteristics of gels of different acrylamide concentration.

Materials and equipment used

Acrylamide, bis-acrylamide (N,N'-methylene-bis-acrylamide and TEMED (N,N,N',N'-tetramethylethylenediamine) were obtained from Sigma. Tris (tris (hydroxymethyl)aminoethane, Analar sucrose and bromophenol blue were obtained from BDH, Bristol. Shandon PAGE equipment for rod gels and LKB2001, vertical electrophoresis unit for vertical gels were used.

Solutions required

Solution A: 0.5 M tris-HCl (pH 8.9) containing 0.46% of N, N,N',N'-tetramethylethylenediamine (TEMED).

Solution B:

Final	% acrylamide	% acrylamide	% bis*
	3	6	0.200
	5	10	0.333
	7	14	0.466

* bis is N,N'-methylenebisacrylamide

Solution C:

Ammonium persulphate (10%) - made fresh in distilled water.

To make the electrophoresis gel the following solutions were mixed in the proportions mentioned:

Solution A	2.5 ml
Solution B	5.0 ml
Water	2.5 ml
Solution C	0.05 ml

The mixture was poured into small glass tubes (5 x 120 mm) or large plastic tubes (10 x 250 mm) for rod gels. It was also used to make the vertical gels. After pouring the mixture, water was layered on top.

Polymerisation took place in about 40 minutes but the gels were normally left for 2 to 4 hours before they were used.

Stacking gel:

Stacking gel was used in both small tubes and the vertical gel electrophoresis system to improve resolution of the protein bands.

Stacking gel solutions

Solution A:

0.5 M tris-HCl (pH 8.3) containing 0.46% of N,N,N',N'-tetramethylethylenediamine (TEMED).

Solution B:

Final acrylamide concentration of 3% in distilled water.

The mixture was mixed in the same proportion as electrophorsis gel.

Solution A	2.5 ml
Solution B	5.0 ml
Water	2.5 ml
Solution C	0.05 ml

The stacking gel was usually 10 mm in height.

When polymerised and loaded into the apparatus large rod

gels (10 x 250 mm) were ran at 1.5 mA/tube, small rod gels (5 x 120 mm) at 3 mA/tube and vertical gels at a total of 50 mA.

Before the sample was applied to the gel in 10% sucrose, bromophenol blue (a small grain of dye to give some blue colour) was added so that the migration could be monitored.

Electrophoresis buffer: 0.05 M tris-HCl, pH 8.9.

Staining of the electrophoresed gels (rod and vertical)

Two stains were used in staining polyacrylamide gels:

Rapid staining was achieved by immersing the gels in rapid stain (0.40 g Coomasie Blue G-250 dissolved in a litre of 7% perchloric acid in distilled water) for 10 minutes.

Slow staining was carried out by immersing in Coomasie Blue R-250 (0.1% Coomasie Blue R-250 in 25% methanol, 10% acetic acid, 65% water (v/v/v) and filtered before use). The gels were left in this stain for one hour.

Destaining

After immersion of gels in rapid stain for 10 minutes, stain was poured back into the bottle and gels were immersed in 7% acetic acid for 10 minutes after which

time the protein bands became visible.

After immersion in slow stain the stain was poured back into the bottle and the gels were washed several times with 5% acetic acid in 20% methanol and left in the same destaining solution until the background was clear.

(f).DETERMINATION OF PROTEIN CONCENTRATION (Spector, 1978; Sedmak and Grossberg, 1977).

(i).Coomassie Blue Binding Reagent

60 mg Coomassie Brilliant Blue G-250 (obtained from Sigma) was dissolved in 1 litre 3% perchloric acid, and filtered to remove undissolved material. The absorbance at 465 nm was between 1.3 and 1.5. This solution was stable indefinitely.

Procedure

To 1.5 ml of sample containing up to 50 ug protein, 1.5 ml Coomassie Blue reagent was added and absorbance was read at 595 nm after 2-30 minutes (Sedmak and Grossberg, 1977).

(ii).Protein measurement with the Folin Phenol Reagent (Lowry et al,1951)

Reagent A: 0.5 g copper sulphate ($CuSO_4 . 5H_2O$) and 1.0 g sodium citrate (Na_3citrate) were dissolved in 100 ml

water.

Reagent B: 20 g Sodium carbonate (Na_2CO_3) and 4 g sodium hydroxide were dissolved in 1 liter water.

Reagent C: Reagents A and B were mixed in 1:1 just before use.

Reagent D: Folin-Ciocalteau reagent (obtained from BDH, Bristol) was diluted 1:1 just before use.

Procedure

Standard curve was prepared with protein concentration ranging from 0.05 mg to 0.5 mg protein using bovine serum albumin (B.S.A. obtained from Sigma) as follows:

To 0.5 ml of each protein solution 2.5 ml Reagent C was added and after thorough mixing on a vortex mixer allowed to stand at room temperature for 5-10 minutes.To this mixture 0.25 ml Reagent D was added and mixed well and let it stand 20-30 minutes to develop colour and then read the absorbance at 700 nm.

When unknown samples were used various dilutions were used to suit the standard proteins range. 0.5 ml of unknown solution replaced standard protein solution and reagents were added as above for standards.

A blank was also used as control (usually water).

3. PREPARATIVE ISOELECTRIC FOCUSING AND DETERMINATION OF ISOELECTRIC POINT OF SHEEP ALPHA2-MACROGLOBULIN

Isoelectric focusing was carried out using LKB 8100 Ampholine Electrofocusing Equipment.

(a). PRINCIPLE

When a mixture of low molecular weight carrier ampholites are exposed to a voltage in a convection-free water solution, a pH gradient is formed. Large molecular weight ampholites, such as proteins, will, given these conditions, concentrate in narrow zones. The concentration will occur at the isoelectric point of the protein. The isoelectric point represents a pH value at which an ampholytic substance has net zero charge and does not migrate. This value is individual to each protein species.

(b). COLUMN USED FOR ELECTROFOCUSING: LKB 8101, 110 ml glass column.

(c). SOLUTIONS REQUIRED

Dense electrode solution: Anode at bottom of column

Electrode solution was made up mixing as follows:

Phosphoric acid	0.2 ml
Distilled water	14.0 ml
Sucrose	12.0 g

Light electrode solution: Cathode at top of column

Electrode solution was made up mixing as follows:

Sodium hydroxide	0.1 g
Distilled water	10.0 ml

Solutions for density gradient

Dense solution

LKB carrier ampholytes (3/4 of the total volume to give 1% or 2% concentration) were made upto 42 ml with distilled water and 28 g of sucrose dissolved in this solution.

Light solution

1/4 of the ampholytes remaining were made upto 60 ml with distilled water.

Ampholyte concentration

Concentration of ampholines was chosen as 1% and 2% using 40% ampholines. Total volume required to give the % concentration is mentioned below for the appropriate pH range.

Ampholines used

i. Ampholines pH 3.5 - 10.0 2.5 ml to give 1% conc.

ii. Ampholines pH 3.5 - 10.0 0.5 ml
Ampholines pH 4.0 - 6.0 4.5 ml to give 2% conc.

(d). SAMPLE

5 ml solution of alpha2-M (concentrated Peak I fractions from Sephadex G-200 column) was mixed with 1/4 of the total volume of ampholytes and the total volume was made upto 60 ml with distilled water.

(e). STARTING THE ELECTROFOCUSING

The power supply (LKB 3371) was turned on and the voltage was set to 300. Electrofocusing was carried out for 36 hours. At the end of electrofocusing run the current was constant.

(f). EMPTYING THE COLUMN

The power supply was turned off. Electrode solution from central tube was sucked off to avoid the risk of mixing with the effluent during the emptying process.

Column was emptied by pumping water from the top of the column to force out the contents. 5 ml fractions were collected using Ultrorac II (LKB). Uvicord S was used to monitor the absorbance at 280 nm and pH in each fraction

was measred on a pH meter (Model 291, Pye Unicam).

4. TESTING THE PERMEABILITY OF DIALYSIS MEMBRANE TO ZINC

Dialysis membranes were (obtained from Medicell International Ltd., London) boiled three times in deionised water before use to remove any contaminants present.

Seven dialysis tubes, each containing 2 ml zinc chloride (100 μg Zn/ml) were dialysed against 4 litres of 1 mM tris-HCl buffer, pH 7.4 for 24 hours. One dialysis tube was taken out of the container every 4 hours and zinc was assayed using the atomic absorption spectrophotometer, AA6.

Time of dialysis (hours)	Amount of Zn present as % of starting material
4	50%
8	40%
12	35%
16	15%
20	12.5%
24	4.0%

This experiment showed that the dialysis membrane is permeable to zinc and 96% of the zinc in the dialysis

tubes passed through in 24 hours.

5.RAISING RABBIT ANTI-SHEEP ALPHA2-M ANTIBODIES

Three rabbits were obtained from the Veterinary School, University of Bristol, Langford.

Rabbits numbered 64, 65 and 66 were used to raise the antiserum.

Purified sheep alpha2-M (1 mg/ml) (from large scale method) was diluted in the ratio of 1:3 with Freund's complete adjuvant (Sigma). Initially, 1 ml of this mixture was injected into all four limbs.

Booster injections were carried out with the same protein/adjuvant mixture at 4 week intervals to increase the production of antiserum as following: The amounts used are outlined below.

1st booster injection 1 ml/limb - (only hind limbs)
2nd booster injection 1 ml/limb - (only fore limbs)
3rd booster injection 1 ml/limb - (only hind limbs)

Usually the rabbits were bled 10-14 days after the 3rd booster injection and the plasma was separated for antibody testing.

Following the above bleeding, rabbits were rested for 6

weeks and then 0.5 ml alpha2-M/adjuvant was injected as further boosters as described above. This ensured that the rabbits continued to produce antibodies.

Testing of rabbit plasma for antibodies to sheep alpha2-M

The rabbit antisheep alpha2-M plasma was tested with antigen (sheep plasma alpha2-M). All three (Nos. 64, 65 and 66) plasma samples were tested separately for their antibody specificity by double immunodiffusion technique and immunoelectrophoresis. The procedure adopted was similar to that for testing alpha2-M from purification methods (Section-II,2e).

The two protein peaks from IAC were not separated into two clear peaks and there was an overlapping. Pooled concentrated proteins were a mixture of alpha2-M and IgG.

It is possible to separate this mixture into pure alpha2-M and IgG by using affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia). However, the high cost of the material made it impossible to use. Instead, an IAC method was prefered, as anti-sheep IgG was available in this laboratory for use in IAC. The anti-sheep IgG was coupled to CNBr-Sepharose 4B to purify alpha2-M by separating IgG.

6. IMMUNOADSORBENT CHROMATOGRAPHY OF IgG

This method was essentially as described for alpha2-M purification in Section-II, 2b. Using methods outlined in Section-II, 2b anti-sheep IgG (raised in Bristol Polytechnic) was coupled to CNBr-Sepharose 4B and concentrated protein (all fractions from IAC of sheep plasma, combining Peaks I and II) was applied on the column. Protein fractions were collected at pH 8.0 contained pure alpha2-M due to IgG retention on the column. IgG was eluted with acetate buffer, pH 2.5. Results are shown in Figs.20 & 21 in Section-III, Expt.1.

The above technique demonstrated that sheep alpha2-M can be purified further. However, presence of IgG along with alpha2-M was in no way a hinderance in studying Zn-binding to alpha2-M. It was shown by Prasad (1970) that Zn-binding to IgG appeared to be electrostatic in nature and ^{65}Zn incubated with human IgG the radioactivity did not migrate with the protein peak. The Zn-binding to IgG was tested in this study and no detectable Zn was bound to IgG as shown in Section-II,7. Hence, the above method was not used to further purify alpha2-M. Instead, both IgG and alpha2-M concentrations were determined and the difference in concentration between total protein and IgG gave alpha2-M concentration in the experimental samples.

7. Zn-BINDING TO IgG

(a). Gel electrophoretic analysis

(b). Gel filtration analysis

(a). GEL ELECTROPHORETIC ANALYSIS

Zn-binding to alpha2-M and IgG were tested using sample obtained from IAC on PAGE.

5% polyacrylamide gels were prepared (as described in Section-II,2e,iii) in gel tubes. The following samples were applied mixed with 20% sucrose.

(i). Concentrated sample from IAC (containing sheep alpha2-M and IgG)

(ii). 500 ul concentrated sample (i) mixed with 0.1 uCi ^{65}Zn (0.1 uCi = 0.35 ug Zn) and incubated for 2 hours.

Both (i) and (ii) were mixed with equal volume of 20% sucrose solution in distilled water and 10, 20, 30, 40, 50, 100 and 200 ul were applied to the gels.

After 3 1/2 hours electrophoresis the gel with sample (i) was stained with rapid stain (Section-II,p.67) to fix the position of alpha2-M and IgG bands. The position of these bands were used to locate the protein bands in other gels. These were cut out and their radioactive content measured in a well type Scaler Ratemeter, SR7 (Nuclear

Enterprises).

Results are presented in Appendix-I.

(b).GEL FILTRATION ANALYSIS

Procedure

Zn-binding to IgG using gel filtration consisted four stages of procedure:

(i). Elution of ^{65}Zn

(ii). Elution of IgG

(iii). Elution of IgG incubated with ^{65}Zn

(iv). Calculations

(i).Elution of ^{65}Zn

A concentration of 1500 µg Zn was used to test the Zn-binding to IgG. ^{65}Zn (obtained from Radiochemical Centre, Amersham.) was mixed with cold Zn to obtain final concentration of 1500 µg Zn. This Zn concentration was made upto 200 µl final volume with tris-HCl buffer, pH 7.4 and applied to the columns (Amicon, mini column, 8 x 60 mm) packed with Sephadex G-200 in 5 mM tris-HCl buffer, pH 7.4, containing 0.9% NaCl and 0.02% sodium azide, equilibrated and eluted with the same buffer. A total of 15 fractions of 10 drops (400 µl) collected. Radiactivity count was taken before sample application

and after application in each of the fractions. Percentage count of radiactivity in each fraction was taken and used as blank as explained in (iv).

(ii).Elution of IgG

Stock IgG (Sheep IgG, Product No.I5131: obtained from Sigma); 7.5 mg IgG in 3 ml tris-HCl buffer (i)) was used to obtain 12.5, 25, 50, 75 and 100 μg IgG concentration when made upto 200 μl with tris-HCl buffer as follows:

IgG Stock (μl)	Buffer (μl)	Final IgG concentration (μg/200 ul)
5	195	12.5
10	190	25.0
20	180	50.0
30	170	75.0
40	160	100.0

Each sample was applied to the column (as in (i)) separately. After elution, protein in each fraction was monitored using Coomassie Blue Reagent (Spector, 1978; Sedmak and Grossberg, 1977).

(iii).Elution of IgG incubated with ^{65}Zn

Each IgG solution (as in (ii)) was mixed with each level of Zn (as mentioned below)

IgG Stock (µl)	^{65}Zn (µl)	Buffer (µl)	Final IgG concentration (µg/200 ul)
5	50	145	12.5
10	50	140	25.0
20	50	130	50.0
30	50	120	75.0
40	50	110	100.0

and incubated for a period between 10-30 minutes (Section-II,10), after which time the sample was applied to the column and eluted with tris-HCl buffer.
The radioactivity was determined before application of the incubated sample and after elution of the sample, in each fraction. A total of 15 fractions of 10 drops (400 µl) collected.

(iv).Calculations

Zn bound to IgG was calculated as follows:

Total counts per minute (C.P.M.) upto the elution volume of sample = A

Total C.P.M. when ^{65}Zn alone was applied (upto the elution volume of sample) = Blank = y

Total C.P.M. of bound ^{65}Zn (B) = A-y

C.P.M. of 50 μl ^{65}Zn used in incubation = Z

Z counts per minute = micrograms (C) Zn

B counts per minute = B x C/Z μg Zn

Results are presented in Appendix-I.

8.Zn-BINDING TO ALPHA2-M

Zn-binding to alpha2-M was studied using:

(a). Gel electrophoresis analysis

   (i). Rod gels, 5% (1 x 25 cms)

   (ii). Vertical gel, 5% (0.5 mm thickness)

(b). Gel filtration analysis

(a)ROD GELS, 5%

Rod gel electrophoresis was used to study the influence of 50-400 ug of alpha2-M on Zn-binding to alpha2-M (as shown in Table-III, Expt.2, Section-III).

The method is described in Section-IIe, (PAGE). Rod gels were of bigger size (1 x 25 cms) and gels were run for 24

hours in Buchler PAGE apparatus.

Alpha2-M with a concentration of 1 mg/ml was used as stock and each sample was incubated for 24 hours with 0.1 μCi ^{65}Zn (equivalent to 2.0 μg Zn). Each sample was mixed with 20% sucrose before application to the gel as follows:

Stock alpha2-M (μl)	^{65}Zn (μl)	20% sucrose (μl)
50	100	50
100	100	100
200	100	200
400	100	400

After locating alpha2-M on the gel with rapid stain mentioned in Section-II, p.67), gels were cut and count was taken on Rackgamma 1270 (LKB) and ^{65}Zn bound to alpha2-M was calculated.

(a). VERTICAL GEL, 5%

This technique was used for the following three experiments:

(i). Expt.2 (Table-I) in Section-III, Zn-binding to 25-200 μg of alpha2-M:

Alpha2-M with a concentration of 1 mg/ml was used as stock from which 25, 50, 100, 200 μl were removed and

incubated with 0.1 μCi ^{65}Zn (equivalent to 0.2 μg Zn) for 24 hours. After which time 20% sucrose solution was added to each sample before application to the gel (5% gel was prepared as described in Section-II,2e).

Electrophoresis was carried out at 200V and 45 mA for 10 hours. After electrophoresis alpha2-M was located using rapid stain (Section-II, 2e) and gel was cut and radioactivity count was taken using Rackbeta II(LKB).

(ii). Expt.2 (Table-II) in Section-III, Zn-binding to 50-500 μg of alpha2-M:
5% gel was prepared as described in Section-II,2e. From alpha2-M stock (concentration 1 mg/ml), 50, 100, 200, 300, 500 μl volumes were removed and incubated with 0.1 μCi ^{65}Zn (equivalent to 0.2 μg Zn) for 24 hours. 40% sucrose was added to give a final concentration of approximately 10% sucrose in samples. This was necessary due to limitation of sample slot which could only accommodate 300 μl total volume. However, by using modified spacers it was made possible to accommodate larger volumes. Electrophoresis conditions were similar to the above experiment. Radioactivity count was taken as in (i).

(iii). Expt.4 (Table-VI) in Section-III, combined effect of incubation period and the level of in vitro Cu on in vitro Zn-binding to alpha2-M from sheep plasma.

Alpha2-M was mixed with Cu stock and ^{65}Zn and incubated for 24, 72, 108 and 216 hours. After which time 40% sucrose and distilled water were added and the volumes made upto 200 µl before application to the gel (5% gel was prepared as described in Section-II,2e). Volumes of solutions were as follows:

Alpha2-M (µl)	Cu stock (µl)	^{65}Zn (µl)	D.W. (µl)	40% sucrose (µl)
100	10	10	40	40
100	20	10	30	40
100	30	10	20	40
100	40	10	10	40
100	50	10	-	40

Alpha2-M concentration = 1 mg/ml

Cu stock = 1000 µg/ml

^{65}Zn = 0.1µCi = 0.2 µg Zn

Electrophoresis was carried out at 200V, 45 mA for 10 hours. Radioactivity count was taken as in (i).

(b).GEL FILTRATION ANALYSIS

Gel filtration technique was used in the following experiments to study Zn-binding to alpha2-M.

(i). Influence of increasing amounts of alpha2-M on Zn-binding to alpha2-M (Section-III, Expt.2, Table-IV) in sheep plasma.

(ii). Influence of variable amounts of Zn on Zn-binding to alpha2-M (Section-III, Expt.3, Table-V) in sheep plasma.

(iii). Effect of in vitro Cu levels on in vitro Zn-binding to alpha2-M in sheep plasma (Section-III, Expt.4, Tables-VI & VII).

(iv). Zn-binding to alpha2-M from Cu-deficient sheep plasma (Section-III, Expt.5).

(v). Zn-binding to alpha2-M from high-Cu sheep plasma (Section-III, Expt.6).

(vi). In vitro Zn-binding to alpha2-M from the plasma of three breeds of pregnant ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4 (Section-III, Expt.7).

Procedure

Amicon, mini columns (8 x 60 mm) were packed with Sephadex G-200 in 5 mM tris-HCl buffer, pH 7.4, containing 0.9% NaCl and 0.02% sodium azide and equilibrated with the same buffer. The column rested on fraction collector (Redirac, LKB) arm directly above drop counter.

Zn-binding to alpha2-M was studied at two levels of Zn (150 and 1500 μg). These Zn concentrations were obtained by mixing radioactive Zn (obtained from The Radiochemical Centre, Amersham.) and cold Zn (non-radioactive Zn).

There were four stages of procedure involved as follows:
(i). Elution of ^{65}Zn (both levels),
(ii). Elution of alpha2-M,
(iii). Elution of alpha2-M incubated with ^{65}Zn,
(iv). Calculations.

(i).Elution of ^{65}Zn

50 μl ^{65}Zn of each level was made upto 200 μl with tris-HCl buffer and after radioactivity count was taken using Scaler Ratemeter SR7 (Nuclear Enterprises Ltd.) before application to the gel. After application, sample was eluted with tris-HCl buffer and a total of 15 fractions of 10 drops collected. Counts per minute were calculated for each fraction as a percentage of counts

before application. For each elution a separate column was used to avoid time wastage.

(ii).Elution of alpha2-M

Each sample of alpha2-M was made upto 200 µl with the addition of tris-HCl buffer and applied on an Amicon, mini column packed with Sephadex G-200 and eluted (as in (i)). Elution volume of each sample was measured by monitoring protein absorbance using Coomassie Brilliant Blue reagent (details of the reagent mentioned in Section-II, 2f).

(iii).Elution of alpha2-M incubated with ^{65}Zn

100 µl of each sample was mixed with 50 µl of ^{65}Zn (150 µg Zn and 1500 µg Zn separately) and the volume was made upto 200 µl with tris-HCl buffer. This mixture was incubated for a period between 10-30 minutes at room temperature by shaking gently on a flask shaker. After the radiactivity count was taken the sample mixture was applied to the gel and eluted with tris-HCl buffer. A total of 15 fractions of 10 drops (400 µl) collected. Only the volume equivalent to elution volume of the sample (as in ii) was measured for radioactivity.

(iv).Calculations

The calculations are similar to Section-II,7. However, in experiments iv to vi the concentration of alpha2-M differed and consideration of IgG in the sample was

essential. For this, total protein concentration and IgG concentration were measured first. The difference between total protein and IgG gave the concentration of alpha2-M in the samples from experiments iv to vi. Total protein concentration was determined by Coomassie Blue binding method (Sedmak and Grossberg, 1977). The IgG concentration was determined by single radial immunodiffusion (details in Section-II, 11).

Zinc bound was calculated as μg Zn bound/100 μg alpha2-M, after calculating bound Zn as in Section-II,7.

9. IN VITRO COPPER INFLUENCE ON ZINC BINDING TO ALPHA2-M

The following procedure was used in Expt.4 (Table-VII), Section-III.

Sample incubation procedure

1 ml alpha2-M (1 mg alpha2-M/ml) solution was added to each one of five plastic test tubes and 100, 200, 300, 400 and 500 μl of copper sulphate (500 μg Cu/ml) solution respectively was added to tubes 1 to 5. The tubes were left shaking for 24 hours incubation in cold room at 4° C. After incubation, 110, 120, 130, 140 and 150 μl of the mixture was transfered from 1, 2, 3, 4, and 5 tubes respectively. These volumes of the mixture were equivalent to 100 μg alpha2-M. These volumes were sufficient for 5 incubations each with both levels of Zn.

To each of these tubes 150 and 1500 μg Zn (^{65}Zn + cold Zn) were added separately and after 10-30 minute incubation period at room temperature applied on Sephadex G-200 columns (8 x 60 mm.) and eluted as described in Section-II,7. Zinc bound to alpha2-M was calculated as in Section-II,7.

10. INFLUENCE OF INCUBATION TIME ON ZINC BINDING TO ALPHA2-M

To a fixed volume (100 μl) of alpha2-M, variable quantities of ^{65}Zn (equivalent to 0.7, 1.4, 2.1, 2.8 and 3.5 μg Zn) were added and incubated at room temperature for 10, 20, 30, 40, 50 and 60 minutes. After incubation samples were applied on Sephadex G-200 (8 x 60 cms.) columns, separately, equilibrated with 5 mM tris-HCl buffer, pH 7.4, containing 0.9% NaCl and eluted with the same buffer. Zinc bound to alpha2-M was calculated as described in Section-II,7. Results are presented in Appendix-II, Fig.48.

11. SINGLE RADIAL IMMUNODIFFUSION

(a). PRINCIPLE

Mancini et al (1965) modified Oudins original technique by incorporating the antiserum into thin layer of gel and placing the antigen into wells cut into the gel.

The antigen diffuses radially and a ring of precipitation

forms and moves outwards, becoming stationary at equivalence. The diameter of the ring is a function of the antigen concentration in the well and can be compared to a calibration curve of standard antigens.

IgG (Product No.I5131) was obtained from Sigma Chemical Company, Poole.

(b).PROCEDURE

A glass plate (10 x 20 cms.) was precoated with few drops of agarose (1.5%) solution and placed on a plate leveller. 50 ml of molten 1.5% agarose solution was dispensed into a small test tube in a 56° C water bath.

When the agarose solution reached 56° C, 200 µl of anti-sheep IgG (raised in rabbits at Bristol Polytechnic) was added and mixed well. The mixture was poured onto the precoated glass plate and allowed to set.

After the agarose was set, wells were punched using a 20 µl gel puncher (LKB) and a template.

Stock IgG (1 mg/ml) was diluted to give 5 µg IgG/100 µl and 5, 10, 15, 20 µl volumes (standards) were dispensed into 4 wells and in other wells unknown samples were dispensed in volumes between 5 to 20 µl and the volumes added were made upto 20 ul with saline when less than 20 µl were added in all cases.

The glass plate was placed in a diffusing chamber for at least 48 hours. After which time pressing, drying and staining was carried out as described in Section-II,2e(i).

The diameter (d) of each ring was measured on the stained plate and d^2 of standards was plotted against the concentration of the standards used and the concentration of unknowns were derived by interpolation from the standard curve or specific computer programme.

A typical plate showing single radial immunodiffusion analysis of standards is presented in Fig.5.

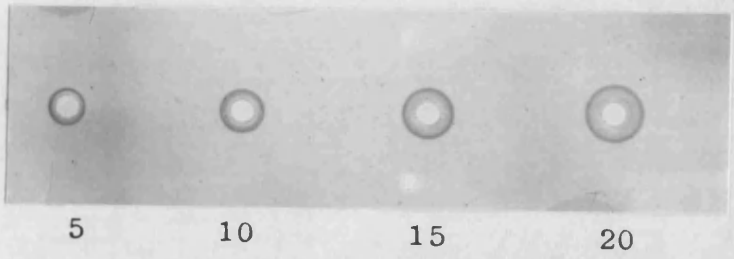


Fig.5 Single radial immunodiffusion of IgG standards.

12. DETERMINATION OF MOLECULAR WEIGHT OF ALPHA2-M BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Molecular weight of nondenatured protein was determined using Sigma Chemical Co.Ltd. kit no. M.W. ND-500.

(a). PREPARATION OF REAGENTS

Separating gel buffer

36.3 g tris(hydroxymethyl) methylamine (tris) and 0.23 ml N,N,N',N'-tetramethylethylenediamine (TEMED) were dissolved in water. The pH was adjusted to 8.9 at 25° C with concentrated HCl and the solution was made upto 100 ml with water.

Stacking gel buffer

5.98 g tris and 0.46 ml TEMED were dissolved in water and pH adjusted to 6.7 at 25° C with concentrated HCl and made upto 100 ml with water.

Acrylamide solution for separating gel

28 g acrylamide and 0.74 g N,N'-methylenebisacrylamide were dissolved in water and diluted to 100 ml.

Acrylamide solution for stacking gel

10 g acrylamide and 2.5 g N,N'-methylenebisacrylamide were dissolved in water and diluted to 100 ml.

Riboflavin solution

4 mg riboflavin was dissolved in 100 ml of water. (Suitable for use in absence of visible microbial growth).

Sucrose solution

5 g sucrose was dissolved in 100 ml of water.

Ammonium persulphate solution

40 mg of ammonium persulphate was dissolved in 5 ml of water. (Prepared fresh).

Electrode buffer

1.2 g tris and 5.76 g glycine were dissolved in water and diluted to 2 litres. The pH was approximately 8.3 at 25° C.

Stored tightly capped at 0-5° C.

Fixative solution

Mixed:

400 ml methanol
70 ml acetic acid
530 ml water

Staining reagent

0.5 g Coomassie Brilliant Blue R-250 was dissolved in 500 ml of fixative solution and stored tightly capped at room

temperature.

Sample buffer

1.0 ml stacking gel buffer, 1.0 ml glycerol and 1.0 ml water containing 0.25 mg bromophenol blue were mixed.

(b).PREPARATION OF SAMPLE

The unknown protein (sheep plasma alpha2-M) was prepared so that the concentration was about 1 mg protein per ml in 5 mM tris-HCl buffer, pH 7.4. The sample was diluted with an equal volume of sample buffer.

(c).PREPARATION OF MOLECULAR WEIGHT MARKERS

Reconstituted each of the protein standard vials with 1.0 ml of 50 mM NaCl-1 mM sodium phosphate buffer, pH 7.0, except urease which was dissolved in 5 ml of water.

Solutions were frozen at -20^{0} C.

Immediately before use, diluted standards with an equal volume of sample buffer.

Standards

Urease , Jack bean	480,000	(tetramer)
	240,000	(dimer)
Albumin, bovine serum	132,000	(dimer)
	66,000	(monomer)

(d).PREPARATION OF ELECTROPHORESIS GELS

The dimensions of gel tube were, 0.5 cm inner diameter and 12.0 cms length. Gels were formed in 20-30 minutes.

(e).PREPARATION OF SEPARATING GELS

Mixed separating gel buffer, acrylamide solution for separating gel, ammonium persulphate and sucrose solution according to the following Table. The mixture was deaerated for one minute with a water pump.

%Gel conc.	4.5	5.0	5.5	6.0	7.0
ml of reagent					
Separating gel buffer	1.5	1.5	1.5	1.5	1.5
Acrylamide sol. for separating gel	1.93	2.15	2.36	2.57	3.0
Sucrose sol.	7.82	7.64	7.40	7.17	6.75
Ammonium persulphate	0.75	0.75	0.75	0.75	0.75

Solutions were mixed carefully to avoid introducing air

and then carefully dispensed 1.75 ml of the mixture into each gel tube as follows:

% Acrylamide	Number of gels
4.5	2
5.0	3
5.5	2
6.0	2
7.0	3

Approximately 0.05 ml of water was layered on top of gel solution, taking care not to disturb the surface of the gel solution and allowed 30 minutes for complete polymerisation.

(f).PREPARATION OF STACKING GEL

To prepare the stacking gel solution, the following solutions were mixed:

2 ml stacking gel buffer
4 ml acrylamide solution for stacking gel
2 ml riboflavin solution
8 ml sucrose solution

Above mixture was deaerated for one minute using water pump. The water layer was removed from polymerised separating gels and top of each of the separating gels was washed twice with approximately 0.2 ml of stacking

gel solution. 0.2 ml of stacking gel solution was carefully dispensed into each tube. After which, approximately 0.05 ml of water was layered carefully on top of the gel solution. The gels were polymerised on the bench in approximately 30 minutes. After polymerisation the gels were used immediately or stored overnight (no longer than 24 hours) at 0-5°C, immersed in water.

(g).ELECTROPHORESIS

Water from the top of gel was decanted and the top of gel was rinsed with 0.2 ml of electrode buffer twice. After which layered electrode buffer on top of each gel to fill tube. Samples were underlayed on gels according to amounts listed below.

Albumin, bovine serum	15 µl
Urease	100 µl
Unknown (alpha2-M)	100 µl

Compartments of electrophoresis apparatus were filled with electrode buffer and electrophoresis was started. A constant current of 1 milliamp per gel was applied until marker dye (bromophenol blue) was completely through the stacking gel (about 1/2 to 1 hour); then increased to 2 milliamps per gel until the marker dye was one centimeter from the anodic end of the gel (about 3 hours).After which time power supply was switched off. The gels were removed from tubes by injecting water between the gel and

the glass wall with a syringe, then exerted pressure at the end of the tube with a pipette bulb. The migration of bromophenol blue dye (centre of the band) was measured and noted.

(h).STAINING AND DESTAINING

The gels were immersed in fixative for about 2 hours, after which time the gels were left in staining solution overnight. The gels were destained in fixative solution by diffusion against several changes of fixative solution (no longer than 24 hours). Then the gels were transfered into 7% acetic acid solution for storage. The gels were allowed to stand in acetic acid solution for at least three hours before migration distances were read. The migration distances of the tracking dye and of the blue protein bands were recorded from top of the separating gel.

(i).RESULTS

To determine the Relative Mobility (Rf) of a protein, divided its migration distance from top of the separating gel to the centre of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

$$Rf = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

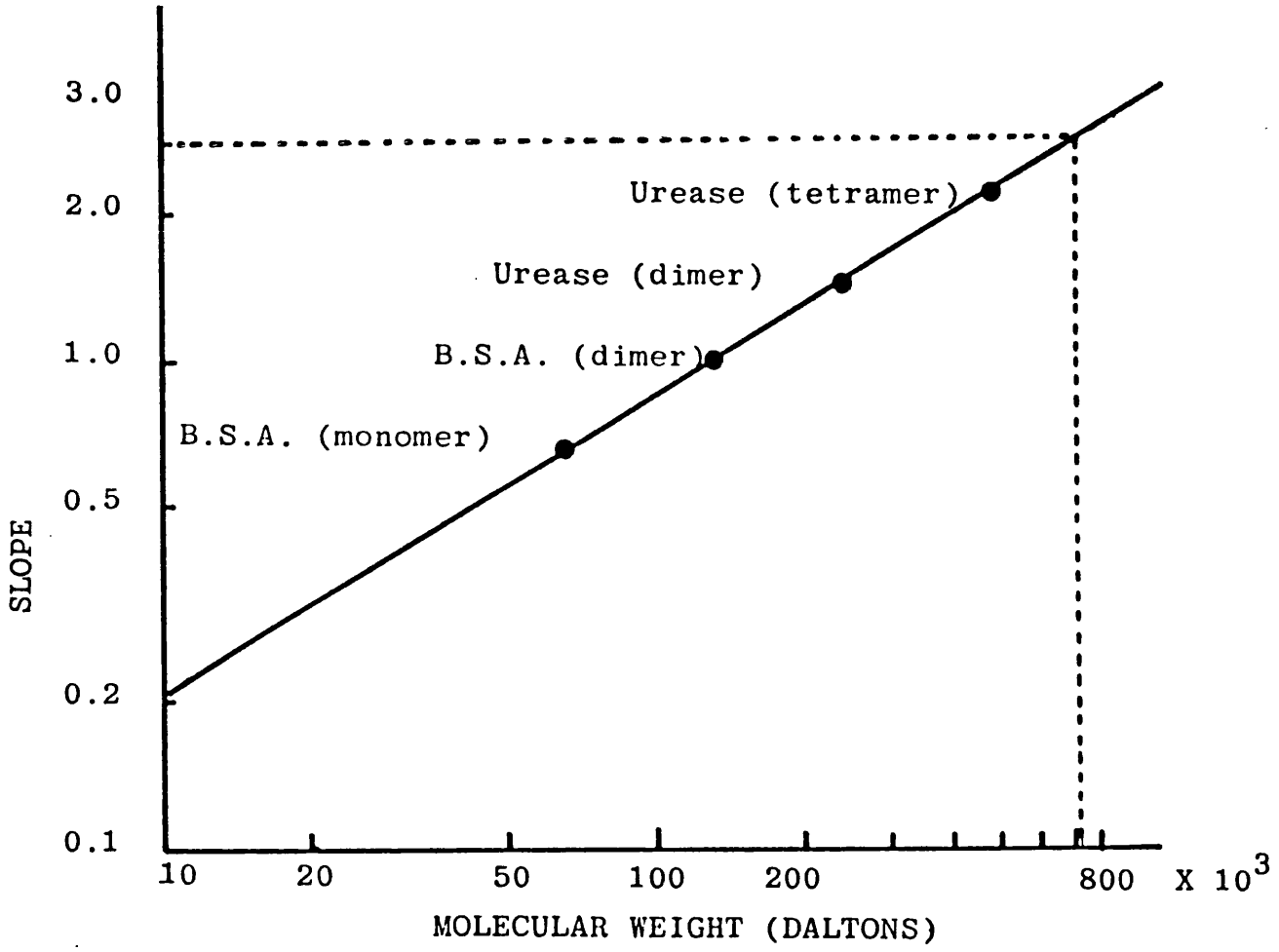


Fig.6 Plot of slopes of standard proteins (Urease,Jack bean (tetramer), 480,000; Urease,Jack bean (dimer), 240,000; albumin,bovine serum (dimer), 132,000 and albumin,bovine serum (monomer), 66,000 daltons) obtained after non-denatured protein PAGE) against molecular weights.

100(log(Rf x 100)) values (ordinate) were plotted against the gel concentration as percent (abscissa) on standard graph paper for each protein. The negetive slopes from these graphs (ordinate) were plotted against the known molecular weights of the standards (abscissa) on 2 cycle log-log paper. The molecular weight of the unknown protein (sheep alpha2-M) was determined from the graph (Fig.6).

13. DETERMINATION OF MOLECULAR WEIGHT OF ALPHA2-M BY GEL FILTRATION ON SEPHADEX G-200

Procedure

Sephadex G-200 column was equilibrated with 5 mM tris-HCl buffer, pH 7.4 and then 10 ml of Blue Dextran solution was applied on the column. 10 ml fractions were collected and the absorbance was monitored at 280 nm with Pharmacia, UV-2 monitor.

After the determination of void volume from Blue Dextran elution, 10 ml solution of molecular weight markers (Thyroglobulin, 669,000; apoferretin, 443,000; beta-amylase, 200,000; alcohol dehydrogenase, 150,000; and bovine albumin, 66,000 were obtained from Sigma Chemical Co.,Poole.) mixture was applied on the same column and they were eluted with tris-HCl buffer, pH 7.4 and 10 ml fractions were collected. Calibration curve of

log molecular weight against Ve/Vo was drawn as shown in Fig.8 in Section-III.

Protein from large scale purification procedure (after centrifugation) was passed through the above Sephadex G-200 column and after calculating Ve-V0, the molecular weight was read from the calibration curve as shown in Fig.8, Section-III.

14.SEPARATION OF ALPHA2-M AND ALBUMIN ZINC ON AFFI-GEL BLUE

(Technical Bulletin 1049E)

Materials required:

Affi-Gel Blue, 50-100 mesh (Bio-Rad Laboratories Ltd.),Whatman column (1 x 10 cms.), 0.02 M phosphate buffer, pH 7.1 (Buffer A), Dialysis tubing, 1.4 M NaCl in buffer A (Buffer B) and 8M urea in buffer A.

Affi-Gel Blue (Bio-Rad Laboratories, Ltd.) selectively adsorbs albumin from serum or plasma. This binding is so strong that a high concentration of salt or chaotropic reagent is required to desorb the albumin. An Affi-Gel Blue column thus provides a simple first step in the purification of serum proteins by removing the major serum constituent, albumin. This specific property of the gel was used to separate zinc bound to albumin and zinc bound to alpha2-M pool in plasma of experimental samples.

The review of literature has shown that zinc is bound to albumin and alpha2-M in significant amounts and a minor proportion to amino acids. All the zinc bound to alpha2-M pool proteins was mainly considered as alpha2-M bound zinc. Copper deficient and copper excess sheep plasma were subjected to chromatography on Affi-Gel Blue.

Procedure for removing albumin from sheep plasma

Plasma sample was dialysed overnight against Buffer A. A Whatman column was packed with Affi-Gel Blue, with a total bed volume of 5 ml per millilitre of plasma. The column was washed with 2 bed volumes of Buffer A and then dialysed plasma sample(4 ml) was applied on the column. Once again the column was washed with 2 bed volumes of Buffer A. The effluent from this wash contained the serum proteins without most of the albumin (Pool-I). The albumin (Pool-II) was eluted with Buffer B. The column was regenerated with 2 bedvolumes of 8M urea in Buffer A.

Wash and elution were monitored for protein absorbance at 280 nm. Only when the absorbance was less than 0.1, buffer was changed after wash to elute albumin. Similarly, when the absorbance was nearer less than 0.1, regeneration of the column was started.

Pool-I and Pool-II fractions were pooled separately and zinc concentration in each was measured on atomic absorption spectrophotometer (AA6).

15. ANALYSIS OF RESULTS

Statistical Package SPSS Rev. 9.1 and Minitab Rev. 5.1 (Copy Right - McGraw-Hill) were used on PRIME 850 computer.

SECTION-III

RESULTS

EXPERIMENT-1

PURIFICATION OF ALPHA 2-MACROGLOBULIN FROM SHEEP PLASMA

INTRODUCTION

Methods are available for the purification of alpha2-M from human plasma, but none are reported for the purification of alpha2-M from sheep plasma. However, Tunstall and James (1974) indicated compatability of anti-human alpha2-M, showing a strong reaction against sheep alpha2-M. This offered an opportunity to adapt the methods used for human alpha2-M purification to sheep alpha2-M purification.

PURIFICATION OF ALPHA2-M

Alpha2-M was purified using two methods, large scale purification method (Section-II, 2a) and immunoadsorbent chromatography method (Section-II, 2b).

A. RESULTS OF LARGE SCALE PURIFICATION

a. GEL EXCLUSION CHROMATOGRAPHY ON SEPHADEX G-200

Elution profile of purified sheep alpha2-M is presented in Fig.7. Chromatography of the final 50 ml extract (Section-II, 2a) showed three distinct peaks and Peak I appeared immediately after Blue dextran Peak (void

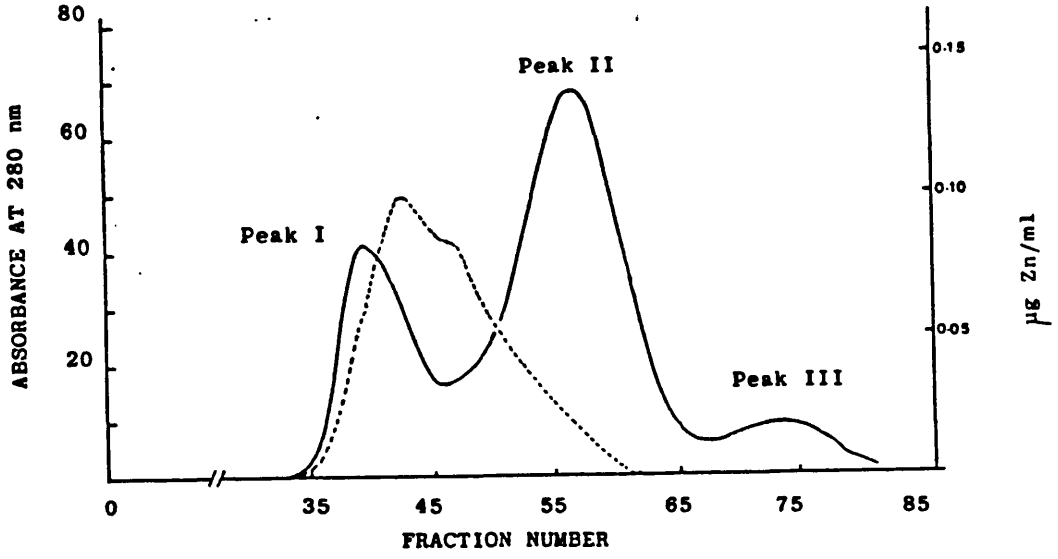


Fig.7 Elution profile of sheep plasma extract (obtained after centrifugation; details in Section-II,2a) on Sephadex G-200 column (5 x 80 cms.); absorbance (smooth line) at 280 nm; concentration of Zn (broken line); 10 ml fractions were collected.

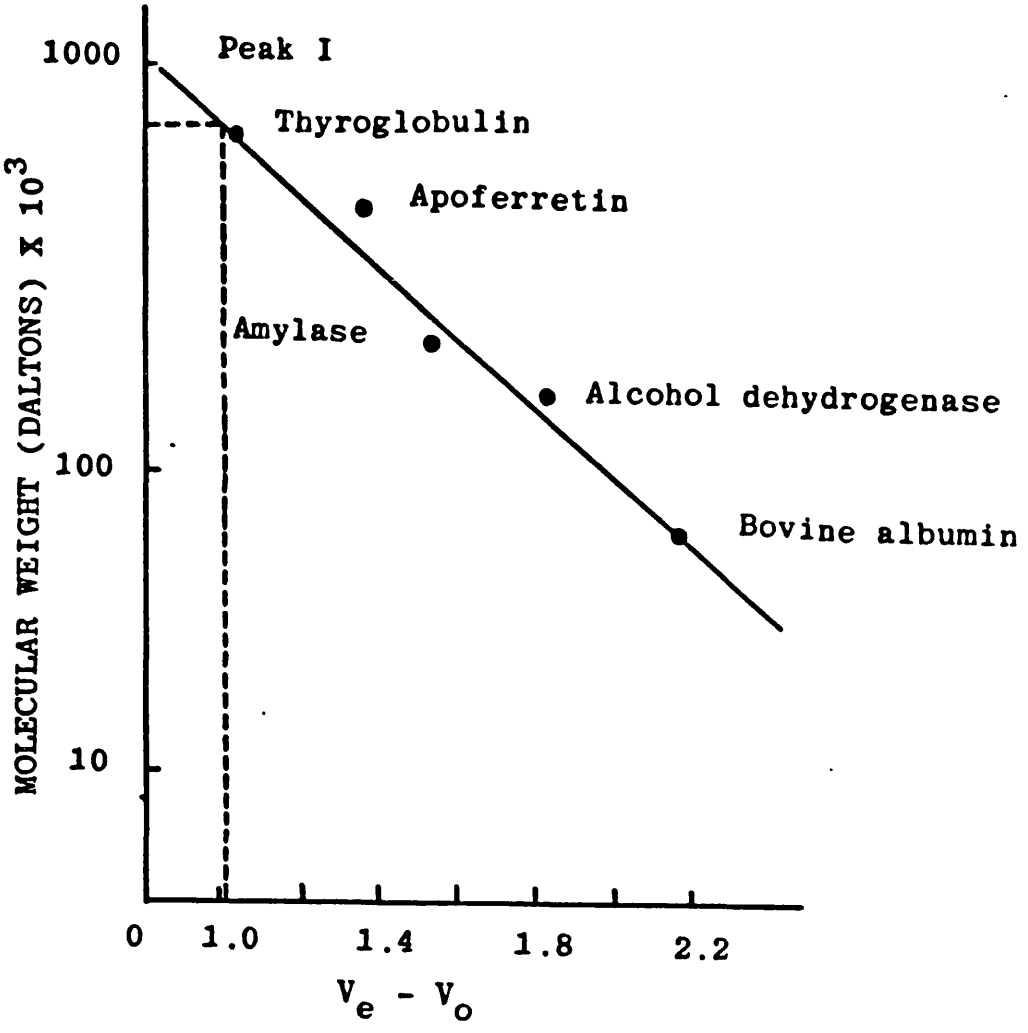


Fig.8 Calibration of Sephadex G-200 column with molecular weight markers.(details on page 101).

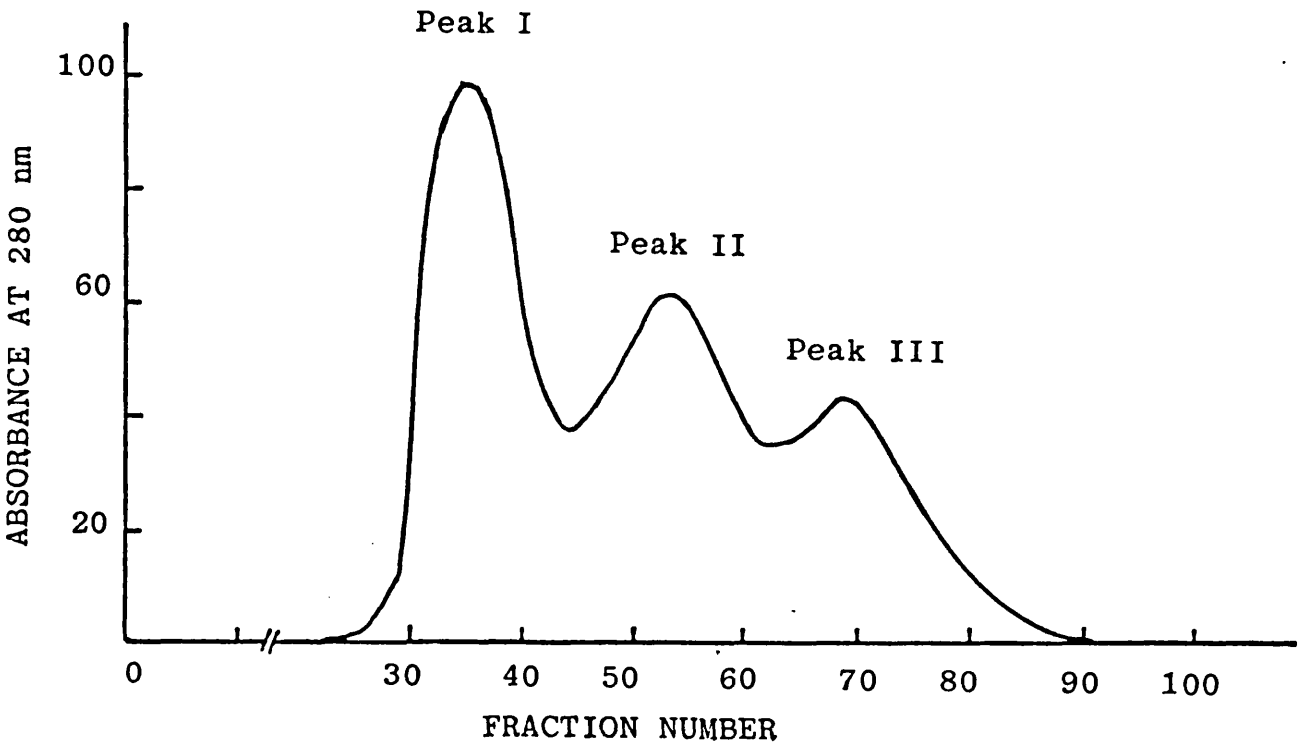


Fig.9 Elution profile of human plasma extract (obtained after centrifugation; details in Section-II,2a) on Sephadex G-200 column (5 x 80 cms.); absorbance at 280 nm. 10 ml fractions were collected.

volume) indicating apparent high molecular weight of the protein. The determination of apparent molecular weights showed Peaks I, II and III as 700,000; 103,000; 45,000 daltons respectively (Fig.8).

Alpha2-M from human plasma was also purified and elution profile (Fig.9) of the final 50 ml extract (Section-II, 2a) showed three peaks with similar molecular weights as above.

b. CHARACTERISATION AND PURITY OF ALPHA2-M

Three methods were employed for this purpose, double immunodiffusion analysis, immunoelectrophoresis and polyacrylamide gel electrophoresis.

These methods are described in Section-II, 2e.

i. Double immunodiffusion analysis

All the eight fractions (Fig.7, fr.37-44) from Peak I showed a single precipitation line (Fig.10) when tested against both anti-human alpha2-M and anti-whole sheep serum confirming the presence of alpha2-M.

The use of anti-whole sheep serum to examine the fractions from Peak I for any protein other than alpha2-M, also confirmed that Peak I consisted of only alpha2-M.

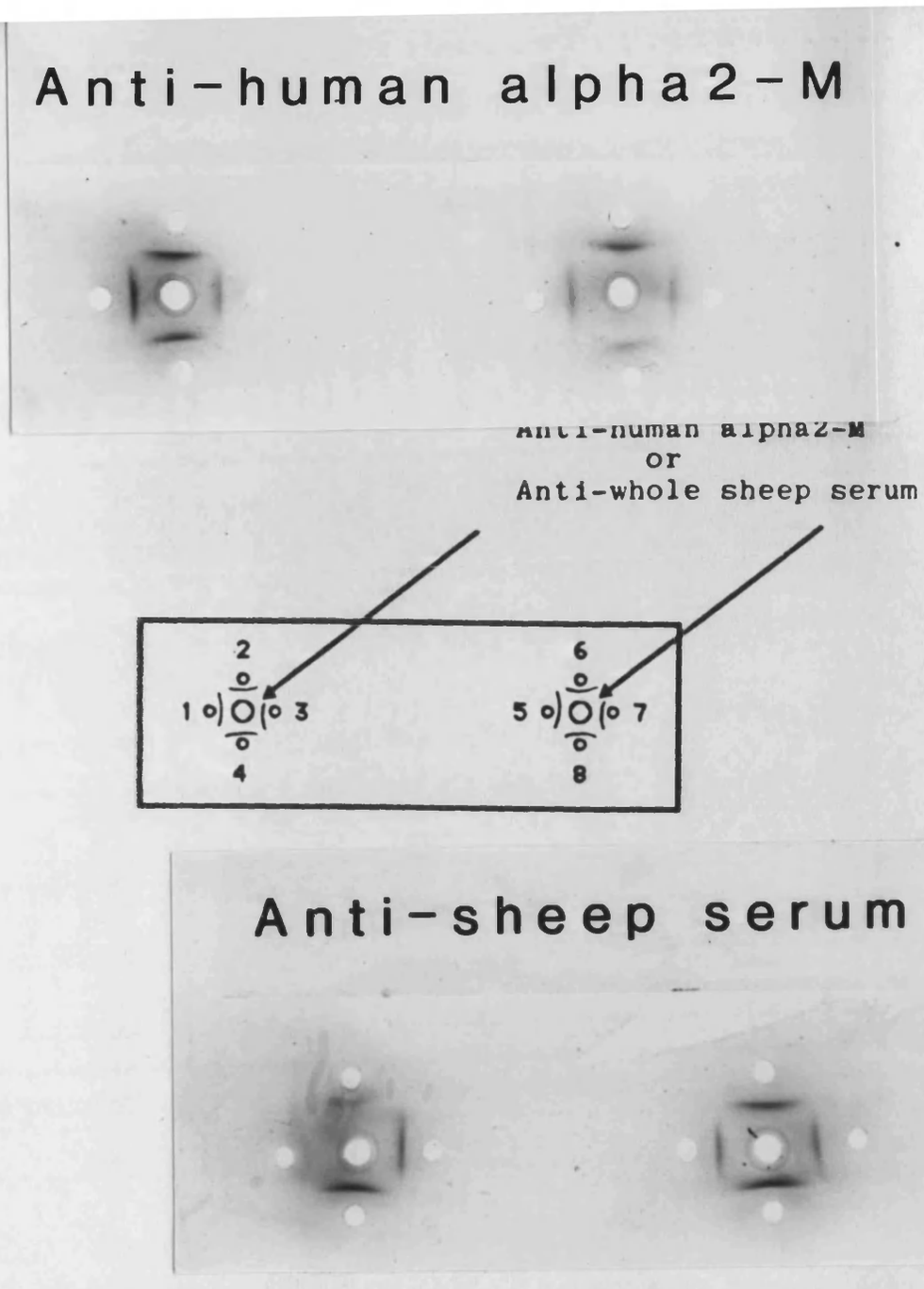
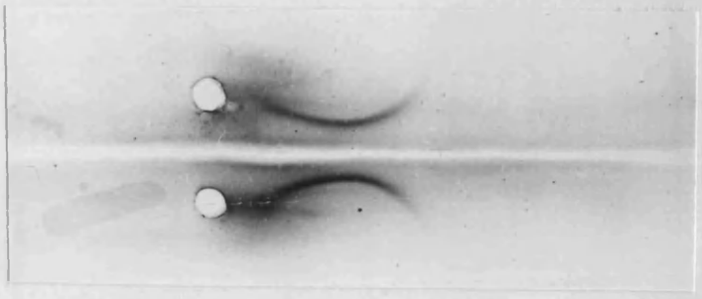


Fig.10 Double diffusion analysis of fractions 37-40 (in positions, 1-4) and fractions 41-44 (in positions 5-8) from Peak I (Fig.7) showing single precipitation lines when anti-human or anti-whole sheep serum was used.



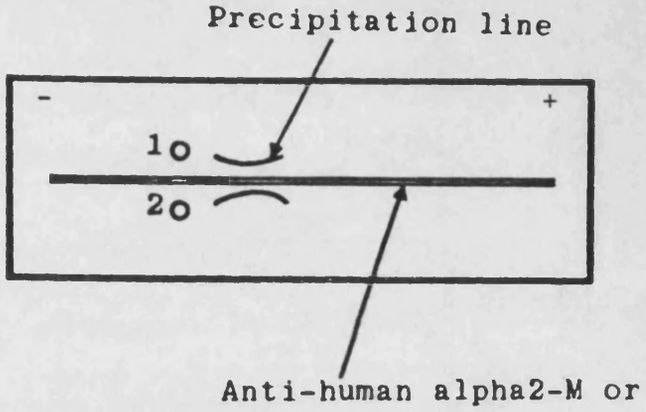


Fig.11 A typical immunoelectrophoresis of fractions from Peak I (Fig.7) showing a single precipitation line when tested against anti-human alpha2-M or anti-whole sheep serum.

Positions 1 & 2 fractions from Peak I (Fig.7)

ii. Immunoelectrophoresis

Immunoelectrophoresis of fractions from Peak I against both anti-human alpha2-M and anti-whole sheep serum confirmed the presence of alpha2-M only (Fig.11).

iii. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of pooled and concentrated fractions from Peak I (gel filtration on Sephadex G-200, Fig.7) on 5% gel, showed a single protein band (Fig.12).

All the three tests above strongly suggested that the fractions from Peak I contained alpha2-M and no other protein, which confirmed that alpha2-M obtained was pure.

c. PRESENCE OF ZINC IN THE PURIFIED ALPHA2-M

Fractions from Sephadex G-200 column (Fig.7) were subjected to atomic absorption to test for the presence of zinc. Peak I fractions and some fractions in Peak II showed the presence of zinc as in Fig.7. Peak I fractions showed up to 0.1 μg Zn/ml in the peak area which gradually reduced towards the end of Peak II. The presence of Zn coincided with alpha2-M. Despite the presence of Zn and alpha2-M in Peak II, due to the presence of other proteins, fractions from this peak were not utilised.

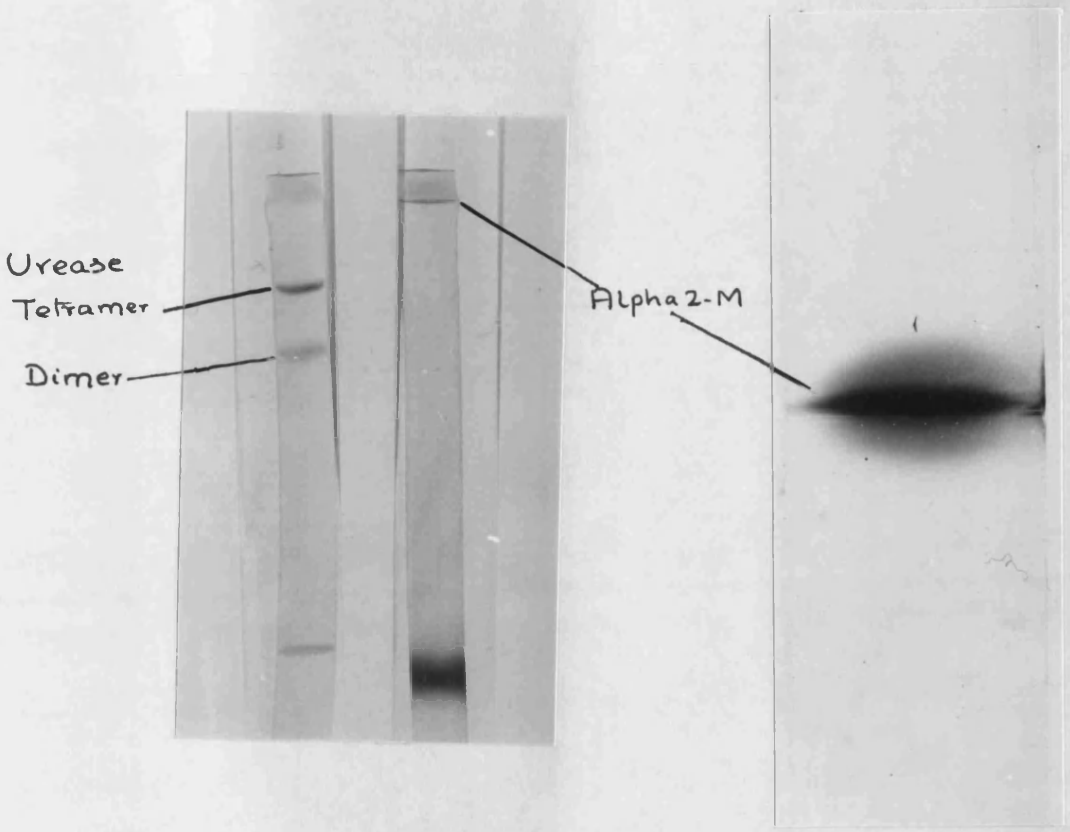


Fig.12 Polyacrylamide gel electrophoresis of concentrated Peak I (Fig.7) on 5% rod gel showing a single protein band which was identified as alpha2-M.

B. IMMUNOADSORBENT CHROMATOGRAPHY (IAC)

IAC was carried out using both anti-human alpha2-M and anti-sheep alpha2-M.

The elution profile presented in Fig.13 shows purification of alpha2-M from sheep plasma using anti-human alpha2-M coupled immunoadsorbent column (Section-II, 2b). Elution with borate buffer, pH 8.0 removed most of the plasma proteins except alpha2-M. Elution with acetate buffer, pH 2.5 yielded a single peak of protein, which was characterised as alpha2-M. These results were similar to the results reported by McEntire (1978) using human plasma. However, alpha2-M purified on anti-human alpha2-M-IAC yielded a single peak first (Fig.13), but after using the column for two or three times two peaks appeared instead of a single peak. The presence of two peaks after IAC, suggested two proteins.

IAC of sheep plasma using the anti-sheep alpha2-M (Section-II, 5) coupled to CNBr-Sepharose 4B showed two protein peaks as shown in Fig.14.

Fractions from IAC were pooled, concentrated and tested to confirm the presence of alpha2-M and the second protein using double diffusion analysis, immunoelectrophoresis and polyacrylamide gel electrophoresis.

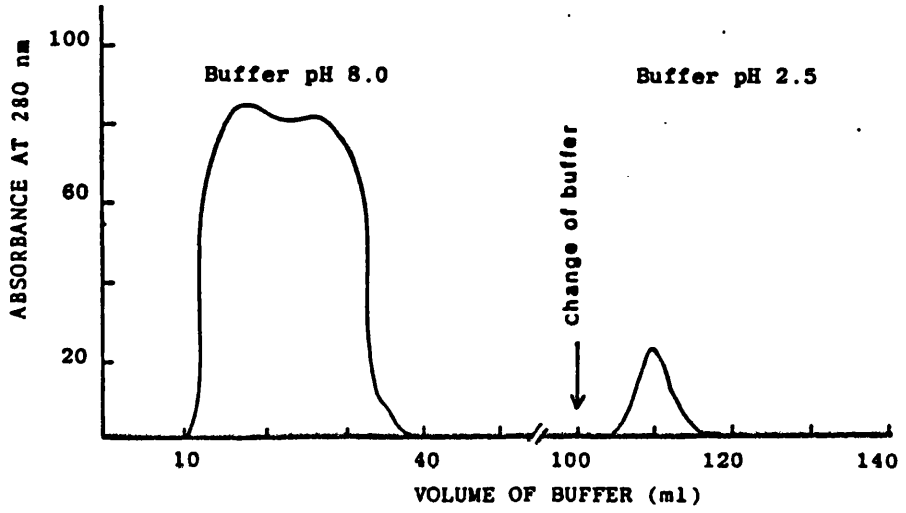


Fig.13 Immunoadsorbent chromatography of sheep plasma on CNBr-Sepharose 4B coupled to anti-human alpha2-M. Sample volume 4 ml.

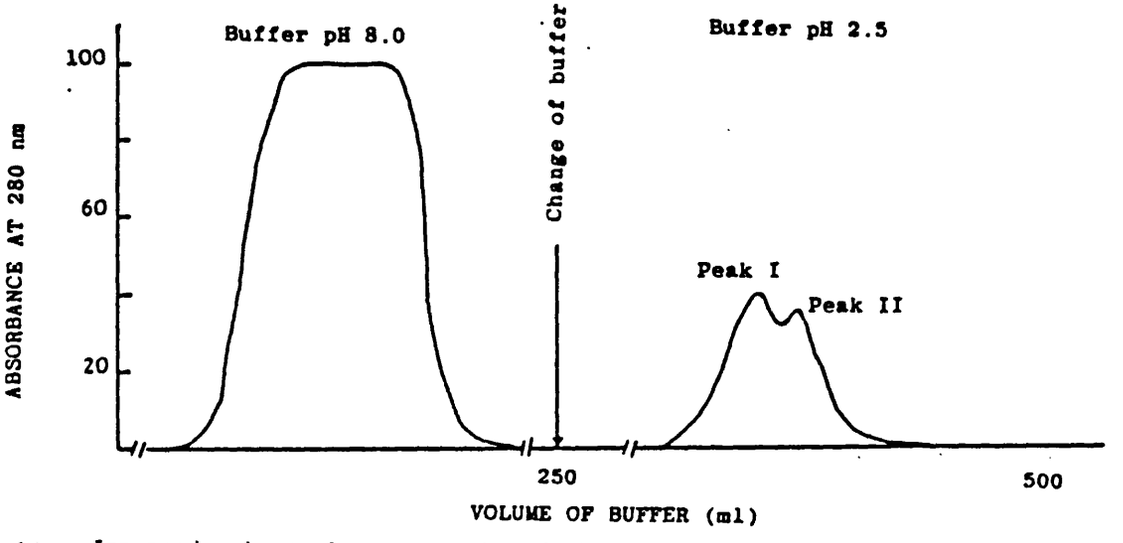


Fig.14 Immunoadsorbent chromatography of sheep plasma on CNBr-Sepharose 4B coupled to anti-sheep alpha2-M, showing two protein peaks after elution with acetate buffer pH 2.5.

i. Double immunodiffusion analysis

Pooled and concentrated protein fractions from IAC columns (a.anti-human alpha2-M coupled, b.anti-sheep alpha2-M coupled) showed a single precipitation line on double diffusion against anti-human alpha2-M (Fig.15) indicating the presence of alpha2-M. However, using antisera against sheep serum showed two precipitation lines (Fig.16) confirming the results obtained on IAC (Fig.14).

ii. Immunoelectrophoresis

Immunoelectrophoresis of pooled and concentrated fractions from sheep plasma IAC also confirmed the presence of two proteins (Fig.17).

iii. PAGE

IAC peaks I and II (Fig.14) were combined and concentrated and then 50 µl of this was applied on 5% PAGE. Results showed two distinct protein bands (Fig.18).

Alpha2-M obtained from gel filtration (Song et al, 1975; Methods, Chapter, IIa) used in raising antiserum was probably contaminated. Among the proteins which were likely to be associated with alpha2-M in sheep plasma , transferrin (Boyett and Sullivan, 1970) was considered first.

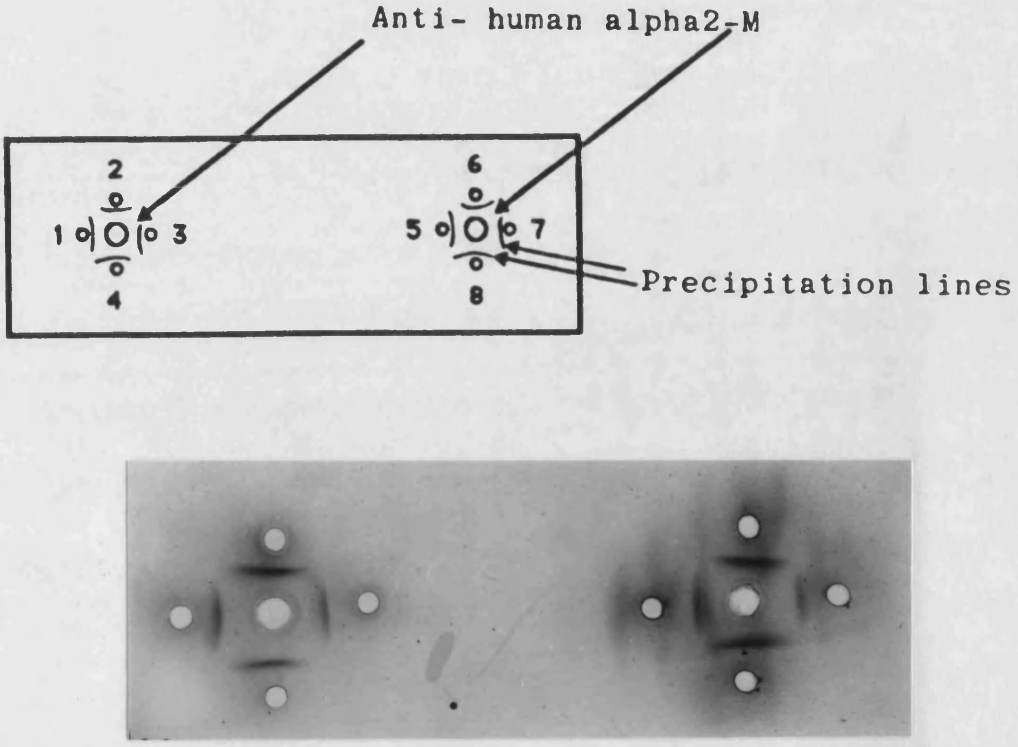


Fig.15 Double diffusion analysis of concentrated fractions, obtained after IAC of sheep plasma on an anti-human alpha2-M coupled CNBr-Sepharose 4B column.

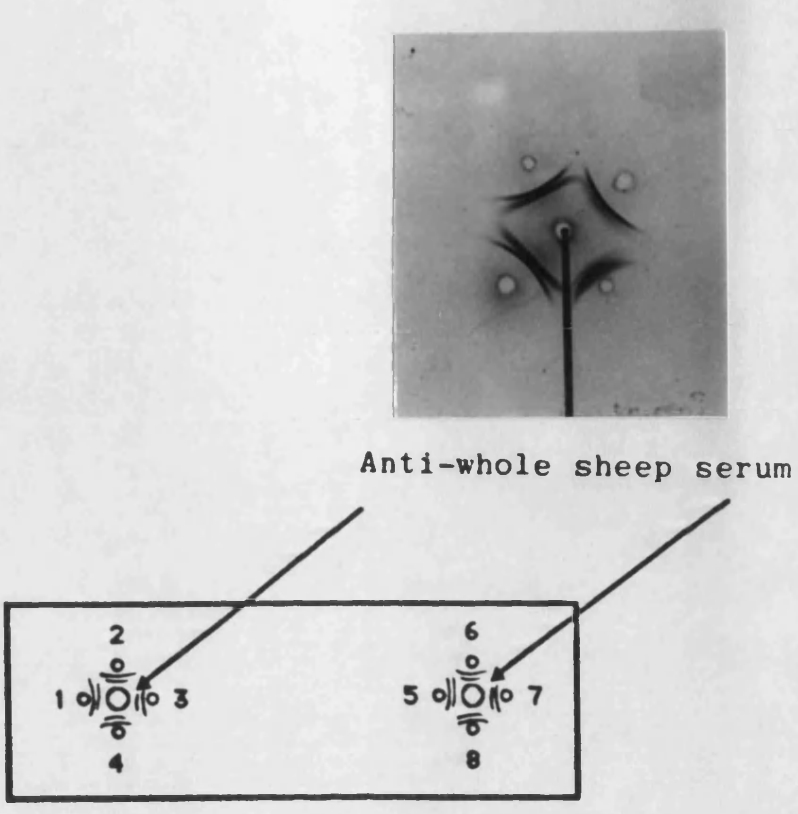


Fig.16 Double diffusion analysis of concentrated fractions,obtained after IAC of sheep plasma on an anti-sheep alpha2-M coupled CNBr-Sepharose 4B column.

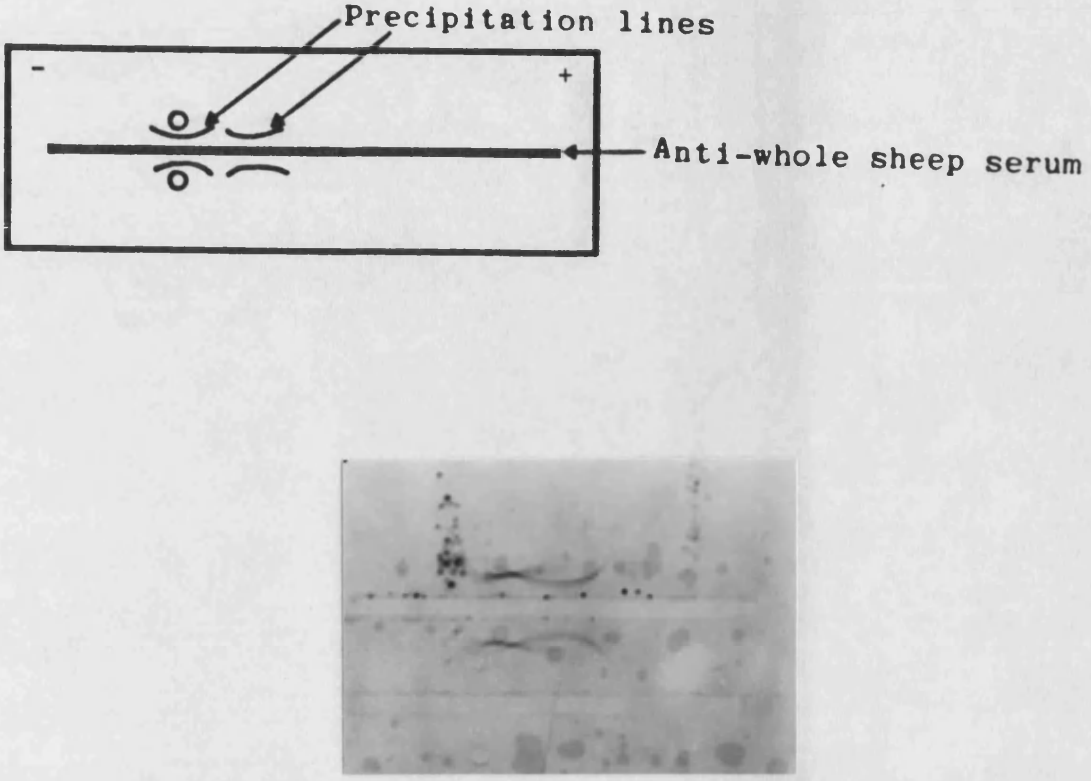


Fig.17 Immunoelectrophoresis of pooled and concentrated fractions from sheep plasma IAC (anti-sheep alpha2-M coupled column) against anti-whole sheep serum, showing two precipitation lines.

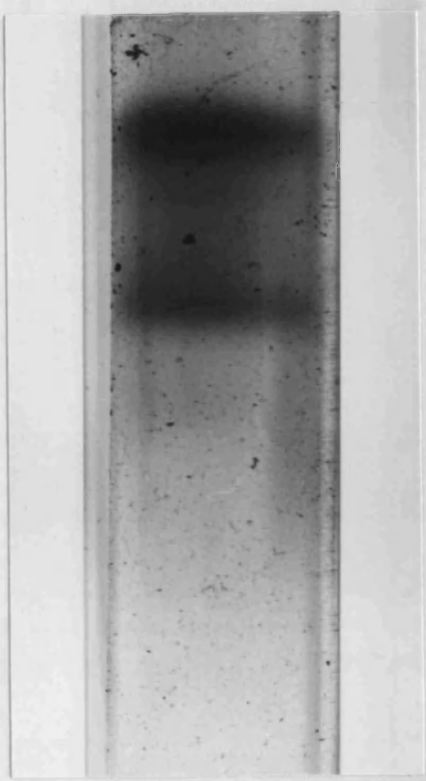


Fig.18 Polyacrylamide gel electrophoresis of pooled and concentrated fractions from sheep plasma IAC (anti-sheep alpha2-M coupled column) on 5% rod gels, showing two protein bands.

The presence of contaminant was observed in concentrated fractions suggested the contaminant present in trace amounts. Hence, concentrated alpha2-M from gel filtration and IAC methods were tested and tests confirmed the presence of a contaminant protein.

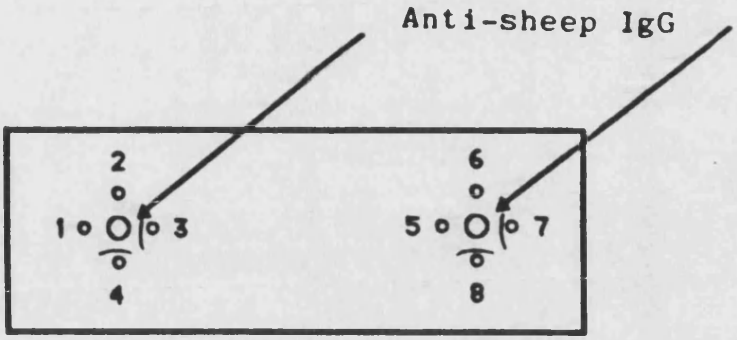
IDENTIFICATION OF PROTEIN CONTAMINANT IN ALPHA2-M

On double diffusion and immunoelectrophoresis of concentrated IAC fractions and gel filtration Peak I against anti-human transferrin, there was no reaction with the contaminant confirming that the contaminant was not transferrin.

Double diffusion of concentrated fractions of IAC and gel filtration Peak I against anti-sheep IgG (raised in rabbits,at Bristol Polytechnic) showed a positive reaction (Fig.19). These tests confirmed the contaminant (2nd protein) in concentrated IAC fractions and Peak I (Fig.7) as IgG.

PURIFICATION OF ALPHA2-M FROM IgG

Contaminated alpha2-M after IAC on an anti-sheep IgG coupled CNBr-Sepharose 4B column yielded alpha2-M in pure form at pH 8.0 and IgG at pH 2.5. Fractions from these peaks were pooled, concentrated and tested against anti-whole sheep serum, anti-human alpha2-M and anti-sheep IgG. Purified alpha2-M and purified IgG showed



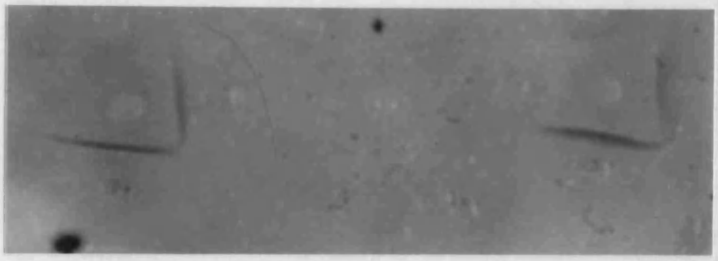
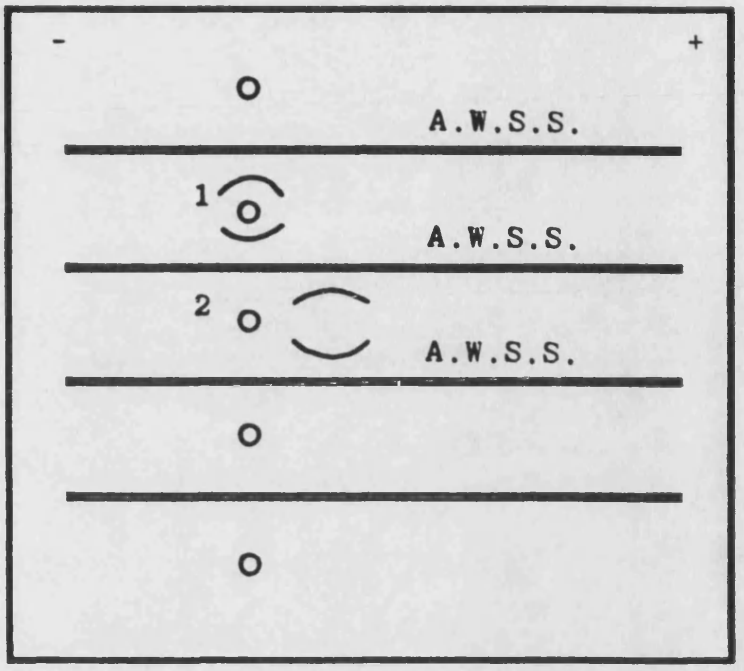


Fig.19 Double diffusion analysis of pooled fractions from IAC and gel filtration, Peak I (Fig.7) against anti-sheep IgG. Antigen in positions as follows:

Unconcentrated, IAC fr.	1 & 5
Unconcentrated, Peak I	2 & 6
Concentrated, IAC fr.	4 & 8
Concentrated, Peak I	3 & 7



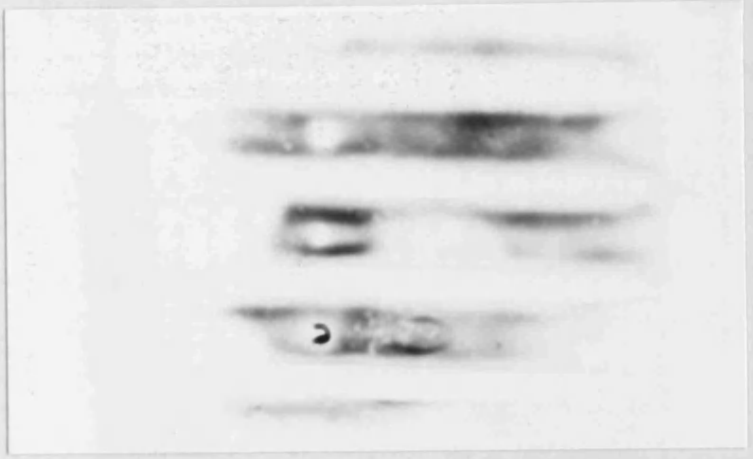


Fig.20 a Immunoelectrophoresis of purified sheep alpha2-M (position 2) and purified IgG (position 1) against anti-whole sheep serum (A.W.S.S.).

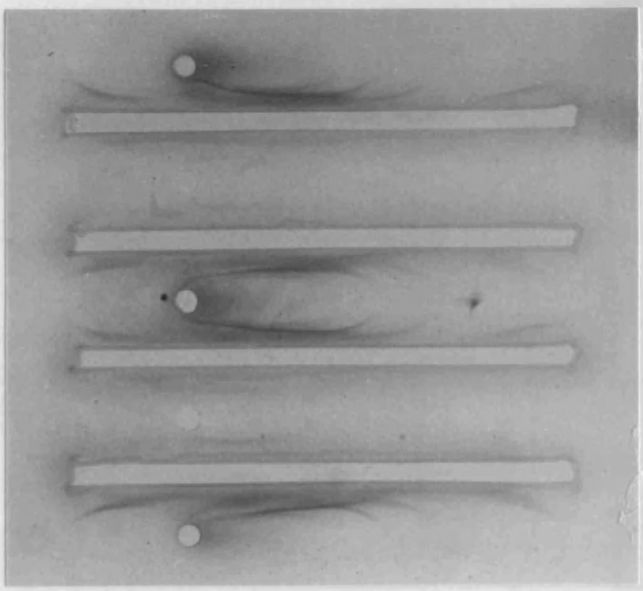
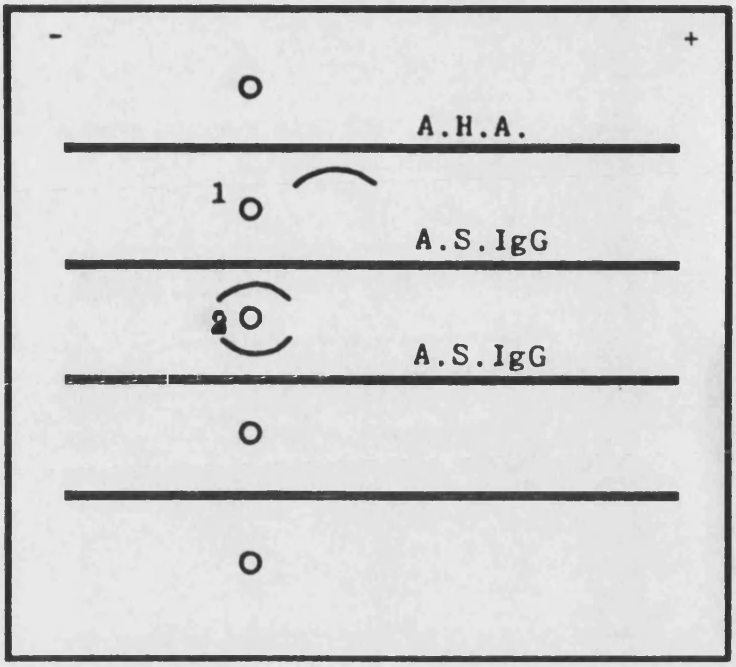


Fig.20b Photograph showing immunoelectrophoresis of sheep IgG and plasma against anti-sheep serum.



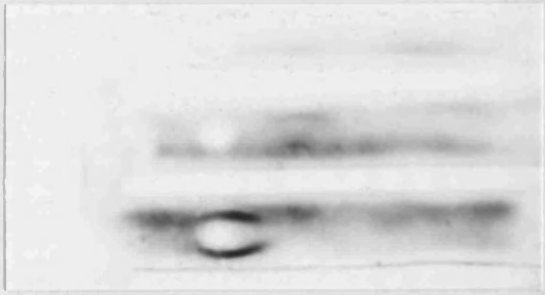


Fig.21 Immunoelectrophoresis of purified sheep alpha2-M (position 1) and purified IgG (position 2) against anti-human alpha2-M(A.H.A.) and anti-sheep IgG (A.S.IgG).

a single precipitation line against each of the anti-sera (Figs.20a and 21). Fig.20b shows a single IgG precipitation line against anti-whole sheep serum compared to several plasma proteins with sheep plasma.

Further purification of alpha2-M using an anti-IgG coupled CNBr-Sepharose 4B was a new modification (Section-II, 6) resulting from these studies to remove IgG when it is present as contaminant associated with sheep plasma alpha2-M.

FURTHER PURIFICATION OF ALPHA2-M BY PREPARATIVE ISOELECTRIC FOCUSING AND DETERMINATION OF ISOELECTRIC POINT

Isoelectric focusing (Section-II, 3) yielded 4 peaks (Fig.22). Peak III was identified as alpha2-M after immunoelectrophoresis (Fig.23a). Peak III from electrofocusing was also subjected to immunoelectrophoresis against anti-whole sheep serum to test the purity of alpha2-M. Results shown in Fig.23b confirmed that alpha2-M was pure.

Isoelectric point of 4.8, was obtained (Fig.22) using two different mixtures of Ampholines (pH 3.5-10 and a mixture of pH 3.5 + pH 4-6, Section-II, 3).

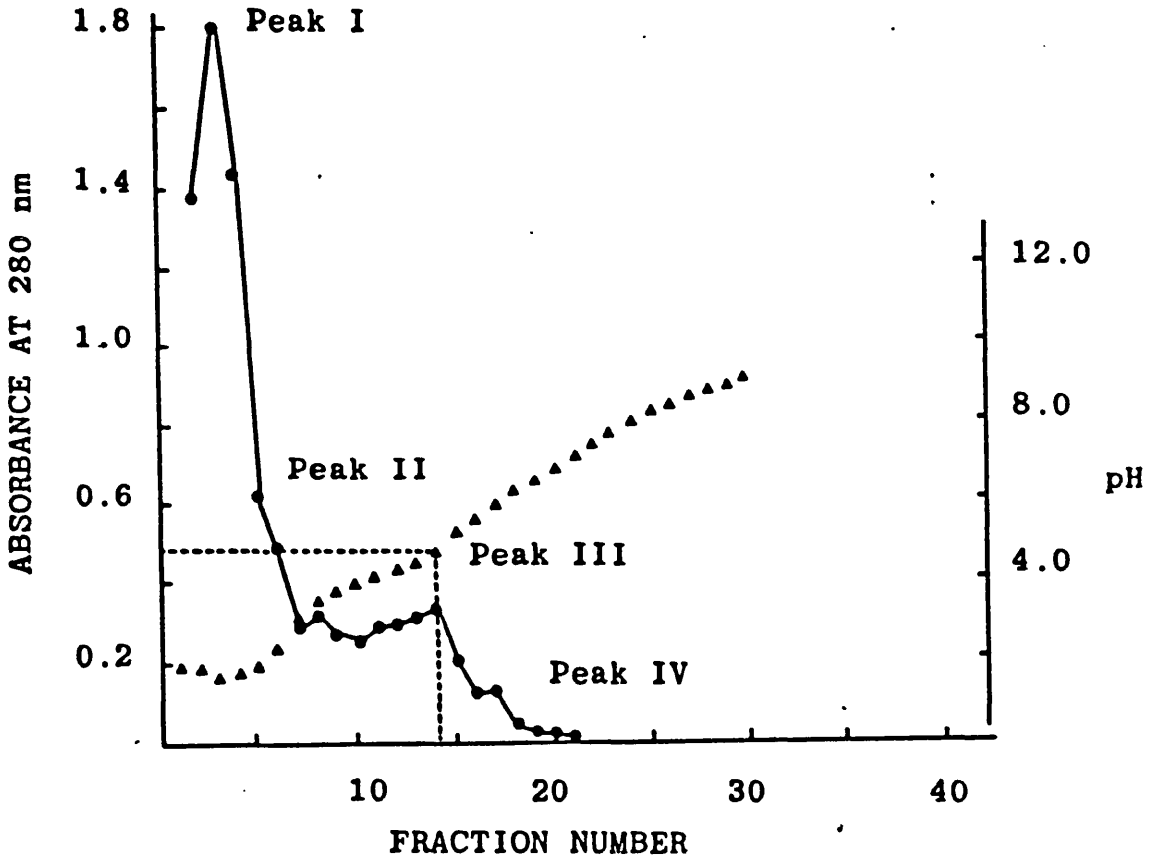
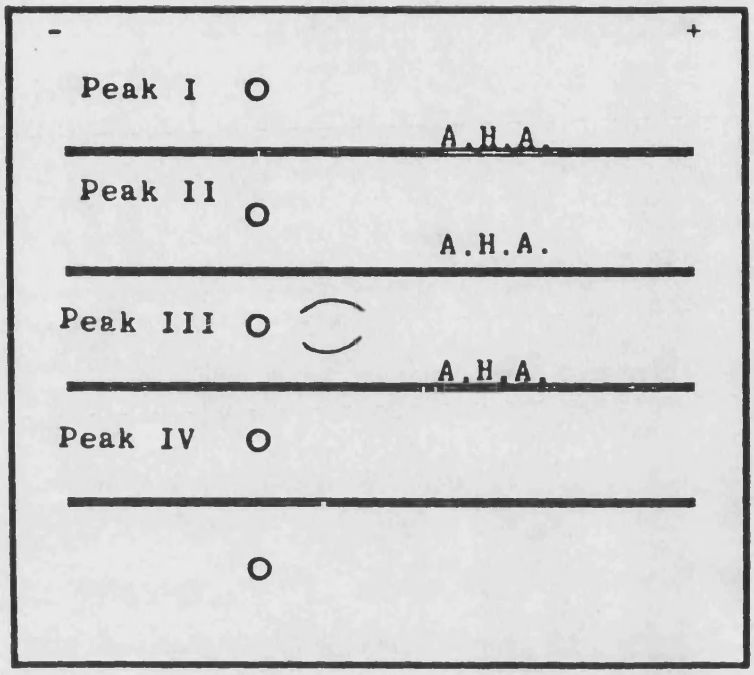


Fig.22 Preparative isoelectric focusing in 110 ml column (LKB). 1% Ampholines (3.5 - 10.0) were used. Voltage applied, 300 V; 5 ml fractions were collected.



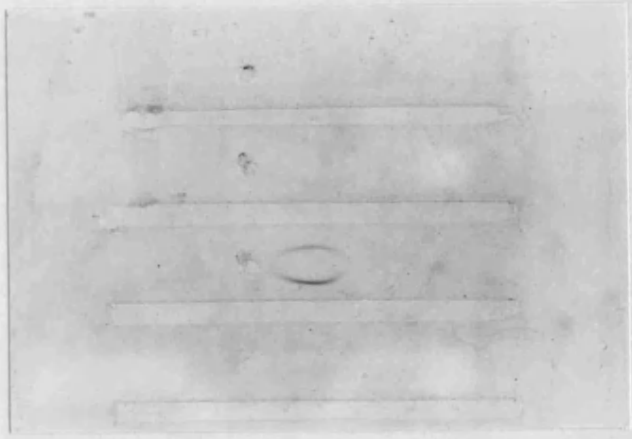
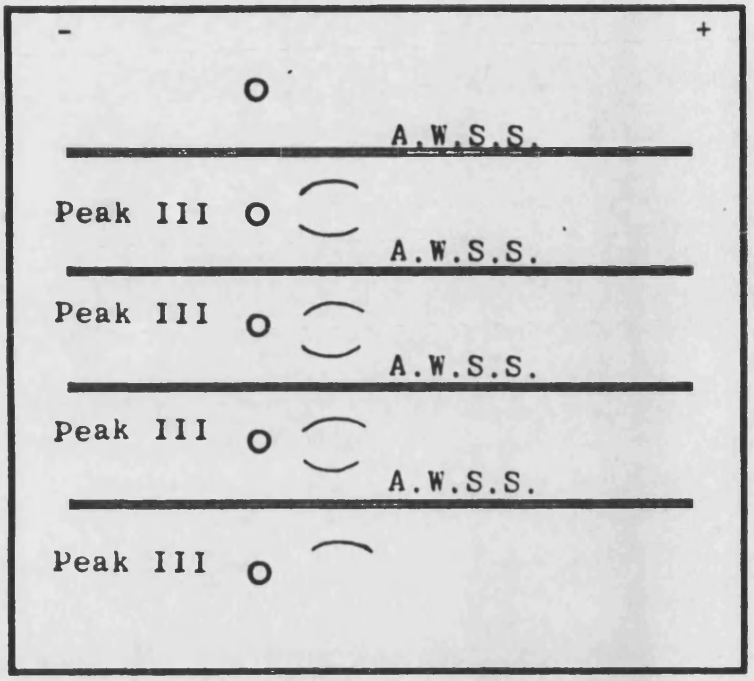


Fig.23a Immunoelectrophoresis of Peaks I, II, III and IV (Fig.22) against anti-human alpha2-M (A.H.A.).



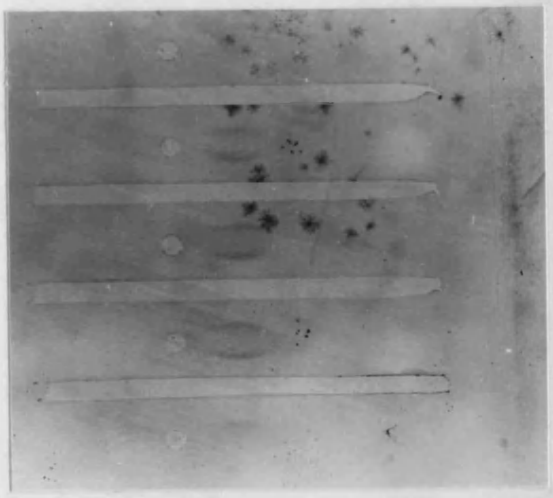


Fig.23b Immunoelectrophoresis of Peak III (Fig.22) against anti-whole sheep serum (A.W.S.S.).

DISCUSSION

Sheep plasma alpha2-M was purified successfully following two methods used for obtaining pure alpha2-M from human plasma (Song et al, 1975; McEntire, 1978).

Adaptation of large scale method (Song et al, 1975) yielded sheep plasma alpha2-M nearer to 100% purity in agreement with Song et al (1975) as in human plasma. However, trace amounts of IgG in association with sheep alpha2-M were discovered only after raising antiserum to sheep alpha2-M, using alpha2-M purified as above (Song et al, 1975).

The presence of transferrin (Boyett and Sullivan, 1970) and beta-globulin (Song et al, 1975) were reported in human plasma alpha2-M. However, association of transferrin with alpha2-M was observed in crude preparations of alpha2-M (Boyett and Sullivan, 1970) from normal and cirrhotic serum. Song et al (1975) reported beta-globulin on immunoelectrophoresis of purified human alpha2-M preparations but not on double diffusion and stressed the importance of immunoelectrophoresis in detecting the presence of contaminants. In this study presence of IgG as a contaminant in purified sheep alpha2-M may be due to the existence of species differences.

In this study alpha2-M purified from both sheep plasma and human plasma showed three protein peaks (Figs.7 & 9). Whereas, Song et al (1975) observed a single protein peak when human plasma was subjected to the same method. Their observations failed to report the appearance of three protein peaks after gel filtration of human plasma extract. These three peaks in human plasma extract differed from sheep. Peak I was dominant in human plasma extract compared to the other two peaks. Whereas, in sheep plasma extract Peak II was dominant followed by Peak I and Peak III was much smaller.

Appearance of IgG as a contaminant in sheep alpha2-M preparations was not reported before. IgG presence in association with alpha2-M suggests stronger attachment in sheep plasma than in human plasma. In view of this it is necessary to take precautions to remove IgG during purification of sheep alpha2-M, particularly with the method of Song et al (1975). However, the presence of IgG could be ignored in Zn-binding studies due to lack of Zn-binding to IgG as shown in Appendix-I.

Among the methods used for the purification of human alpha2-M, the method described by Barrett (1981) dealt with contaminants more thoroughly than other techniques. According to Barrett (1981) a 95% pure human alpha2-M is suitable for many purposes and non-denatured PAGE or immunoelectrophoresis against anti-human serum proteins,

scarcely detects contamination. Barrett (1981) used,immunoadsorbents (CNbr-Sepharose 4B) coupled to all human immunoglobulins, immunoglobulin A, alpha2-pregnoglobulin,haptoglobulin and fibrinogen to remove the remaining 5% other proteins to obtain 100% pure human alpha2-M. However, this is too expensive and in most cases this high purity may not be required.

In this study sheep alpha2-M fractions demonstrated presence of up to 0.1 μg Zn/ml (Fig.7). Song et al (1975) did not measure Zn in the fractions.

Trypsin binding activity of alpha2-M was studied during purification stages by Song et al (1975), but in this study, their method was used primarily to obtain starting material in pure form to raise antibodies to sheep alpha2-M and for preliminary investigations.

Preparative isoelectric focusing was used successfully as a final step for further purification.

In this study electrofocusing of sheep alpha2-M using two different mixtures of ampholines yielded the same pI 4.8. Barrett et al (1979) showed electrofocusing an equal mixture of S and F-forms of human alpha2-M, that both showed the same isoelectric point, 5.2. However, Ohlsson and Skude (1976) reported a large change in pI of human alpha2-M (from 5.0 to 6.0) associated with the complexing

of a proteinase. The differences in isoelectric point between human alpha2-M and sheep alpha2-M may be due to species differences.

EXPERIMENT-2

ZINC BINDING TO ALPHA 2-MACROGLOBULIN IN SHEEP PLASMA

INTRODUCTION

Parisi and Vallee (1970) were the first to report that alpha2-M was a Zn-binding protein.

The concentration of alpha2-M is known to vary between men and women (James et al, 1966a), infants and adults (Ganrot and Schersten (1967), during pregnancy (Ganrot and Bjerre, 1967) and in ataxia telangiectasia, atopic dermatatis, mongolism and diabetes mellitus (James et al, 1966a).

Despite observing variable concentrations of the Zn-binding alpha2-M (Parisi and Vallee, 1970), no published reports have been found which show a relationship between variable concentrations of alpha2-M and Zn-binding.

Therefore studies were carried out to examine the relationship of variable concentrations of sheep plasma alpha2-M on Zn-binding using the techniques as follows:

i. PAGE in 5% vertical gel,

ii. PAGE in 5% rod gels,

iii. Gel filtration on Sephadex G-200.

Details of Zn-binding to alpha2-M using 5% rod gels, 5% vertical gel and gel filtration on Sephadex G-200 are described in Section II,8.

RESULTS

i. Zn-binding to alpha2-M (Table-I and Fig.24) was considered in three stages a. alpha2-M concentrations between 25-200 µg, b. alpha2-M concentrations between 25-50 µg and c. alpha2-M concentrations between 50-200 µg.

Zn-bound to alpha2-M increased from 0.00156 to 0.0110 µg Zn, which was 605% increase (Table-I) corresponding to an increase in alpha2-M concentration from 25 to 200 µg (700% increase), . A 13.0% decrease in g-atoms Zn/mol of alpha2-M was observed during the same change in the concentration of alpha2-M.

Zn-bound to alpha2-M increased by 143.6% when the concentration of alpha2-M was increased from 25 to 50 µg (100%). g-atoms Zn/mol of alpha2-M increased by 21.7% corresponding to alpha2-M increase from 25 to 50 µg (Table-I)

Table- I

A study of increasing alpha2-M concentration on Zn-binding

Alpha2-M (µg)	Bound ^{65}Zn C.P.M.	^{65}Zn bound (µg)	g-atoms Zn/ mol alpha2-M
25	535	0.00156	0.69
50	1293	0.00380	0.84
100	1697	0.00490	0.55
200	3712	0.01100	0.60

^{65}Zn added = 0.1 µCi = 0.2 µg Zn

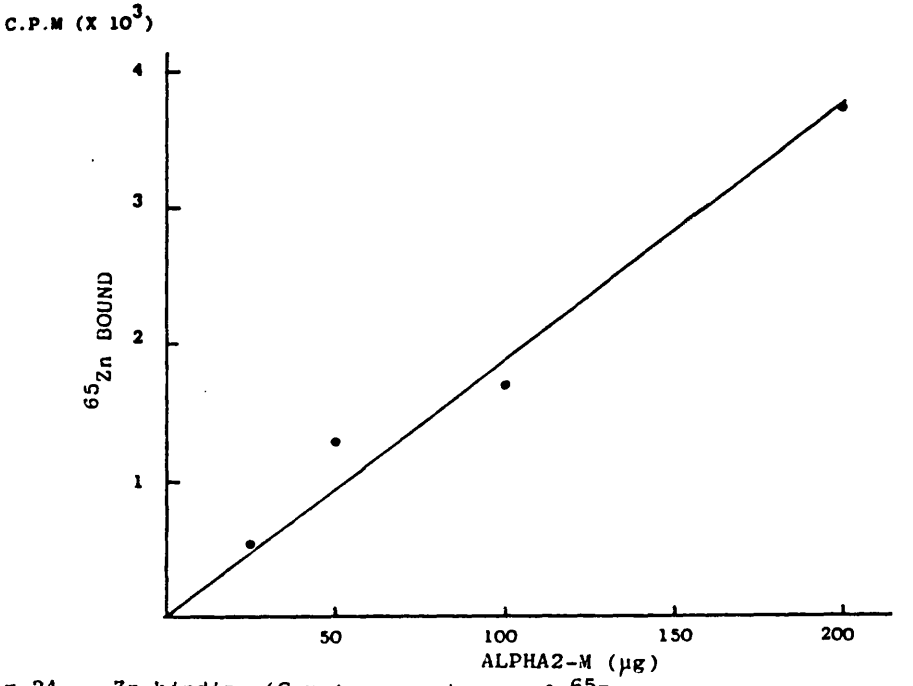


Fig.24 Zn-binding (Counts per minute of ^{65}Zn) to alpha2-M on 5 per cent PAGE (Vertical gel). Constant current, 50 mA; for 10 hours. ^{65}Zn added, 0.1 µCi = 0.2 ug Zn. Alpha2-M incubated with ^{65}Zn for 24 hours at 4° C before application.

Zn-bound to alpha2-M increased by 189.5% in response to an increase in alpha2-M concentration from 50 to 200 μg (300%). Whereas, for this increase in alpha2-M, showed a 28.6% decrease in g-atoms Zn/mol alpha2-M.

Fig.1 shows Zn-binding to alpha2-M increased in a linear fashion from 0.00156 to 0.0110 μg Zn corresponding to alpha2-M increase from 25-200 μg.

At 200 μg of alpha2-M concentration (Table-I), 55 μg of Zn was bound to 1 g of alpha2-M, which was equivalent to 1 g-atom Zn/mol of alpha2-M.

Results in Table-II and Fig.25 showed an increase in μg ^{65}Zn bound, from 0.0136 to 0.0298 (119% increase), corresponding to an increase in alpha2-M from 50 to 200 μg respectively. Corresponding g-atom Zn/mol alpha2-M showed a drop of 45%. This decrease was even more, 89% (from 3.02 to 0.32 g-atoms Zn/mol of alpha2-M) when alpha2-M concentration increased from from 50 to 500 μg. The Zn bound to 500 μg of alpha2-M was only 7.3% higher than that of 50 μg of alpha2-M.

Fig.25 showed that at alpha2-M concentration of 200 μg, maximum concentration of Zn was bound. At this point 149 μg Zn was bound to 1 g of alpha2-M.

At 50 μg of alpha2-M concentration 3.02 g-atoms Zn/mol alpha2-M was observed which was higher than at any other concentration of alpha2-M.

Table-II

A study of increasing alpha2-M concentration on Zn-binding.

Alpha2-M (µg)	Bound ^{65}Zn C.P.M.	^{65}Zn bound (µg)	g-atoms Zn/ mol alpha2-M
50	5908	0.0136	3.02
100	9993	0.0230	2.55
200	12887	0.0298	1.65
300	8706	0.0201	0.74
500	6331	0.0146	0.32

^{65}Zn added = 0.1 µCi = 0.2 µg Zn

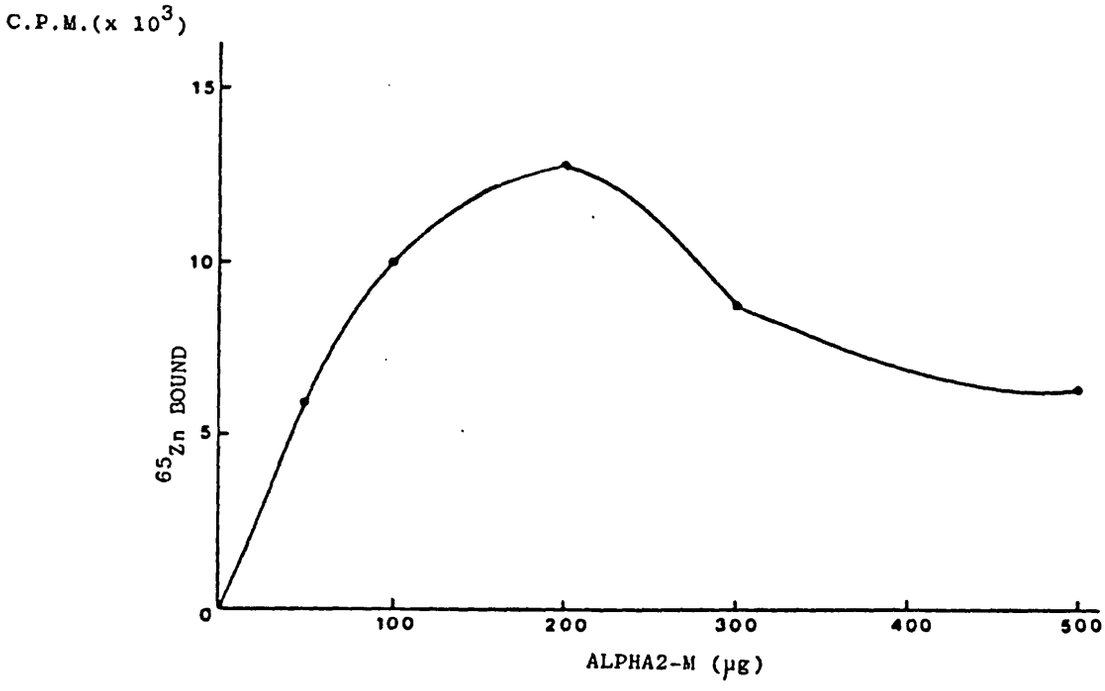


Fig.25 Zn-binding (Counts per minute of ^{65}Zn) to alpha2-M on 5% PAGE (Vertical gel). Constant current, 50 mA; run for 10 hours.

^{65}Zn added = 0.1 µCi = 0.2 µg Zn

Alpha2-M incubated with ^{65}Zn for 24 hours at 4° C before application.

ii. Maximum ^{65}Zn binding (Table-III) in rod gels was observed at at 200 μg concentration of alpha2-M. Increase in alpha2-M concentration from 50 to 400 μg (700% increase) showed 38.8% increase in μg ^{65}Zn bound (0.85 to 1.18 μg Zn), but g-atom Zn/mol of alpha2-M showed a decrease of 82.7%. At the point of maximum Zn binding (Table-III & Fig.26) 5.9 mg of Zn was bound to 1 g of alpha2-M. It appears that from 200 to 400 μg alpha2-M concentration a steady state of Zn binding or equilibrium was achieved.

The results in Table-III demonstrate increased Zn-binding to alpha2-M with increase in alpha2-M concentration. However, at molar level, g-atoms Zn/mol of alpha2-M decreased from 188.70 to 32.70, when alpha2-M was increased from 50 to 400 μg, indicating distribution of Zn to all available Zn-binding sites.

Table-III

A study of increasing alpha2-M concentration on Zn-binding.

Alpha2-M (µg)	Bound ^{65}Zn C.P.M.	^{65}Zn bound (µg)	g-atoms Zn/ mol alpha2-M
50	6685	0.85	188.70
100	8147	1.04	115.35
200	9267	1.18	65.44
400	9271	1.18	32.70

^{65}Zn added = 1.0 µCi = 2.0 µg Zn

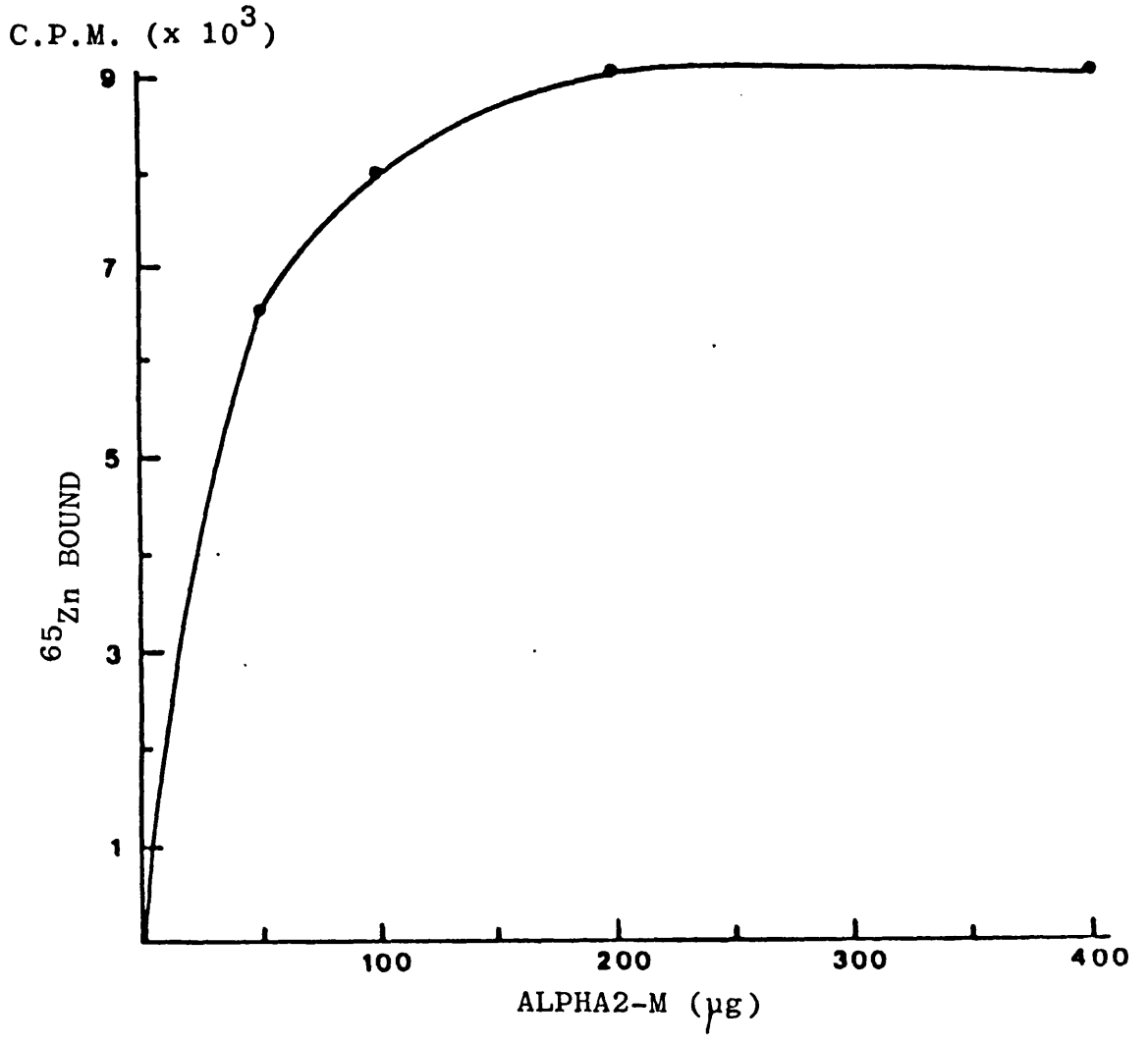


Fig.26 Zn-binding (Counts per minute of ^{65}Zn) to alpha2-M on 5 per cent (rod gels, 1 x 25 cms.) PAGE for 24 hours at constant current, 1.5 mA/ tube. ^{65}Zn added, 1.0 µCi = 2.0 ug Zn. Alpha2-M incubated with ^{65}Zn for 24 hiurs at 4^{o} C before application.

iii. A STUDY OF INCREASING ALPHA2-M CONCENTRATION ON Zn-BINDING (GEL FILTRATION)

Results presented in Table-IV and Fig.27 indicated a significant ($P<0.001$) decrease throughout in Zn-binding to alpha2-M as the concentration of alpha2-M incresed from 50 to 250 μg. From a mean of 200.30 μg Zn bound/100 μg alpha2-M reduced to a mean of 7.20 μg Zn/100 μg alpha2-M. On a weight basis g-atoms Zn/mol of alpha2-M reduced from $222.16.10^2$ to $7.98.10^2$.

Comparison of Zn-bound to 50 and 250 μg of alpha2-M (Table-IV), showed a decrease of 96.4% at 250 μg alpha2-M and most of the decrease observed (78%) was at 100 μg alpha2-M concentration. Percentage decreases between 100-150, 150-200, and 200-250 μg were 47.6, 21.5 and 60.4 respectively.

Table- IV

Zn- binding to 50- 250 µg of alpha2-M incubated with 1500 µg Zn.

µg Zn Bound / 100 µg alpha2-M

Alpha2-M (µg)	Mean	S.E.M.	g- atoms Zn/ mol alpha2-M
50	200.30 ***	± 14.80	$222.16.10^{2}$
100	44.30 ***	± 4.40	$49.13.10^{2}$
150	23.20 **	± 3.20	$25.73.10^{2}$
200	18.20 ***	± 1.90	$20.19.10^{2}$
250	7.20 ***	± 0.50	$7.98.10^{2}$

** $P < 0.01$ *** $P < 0.001$

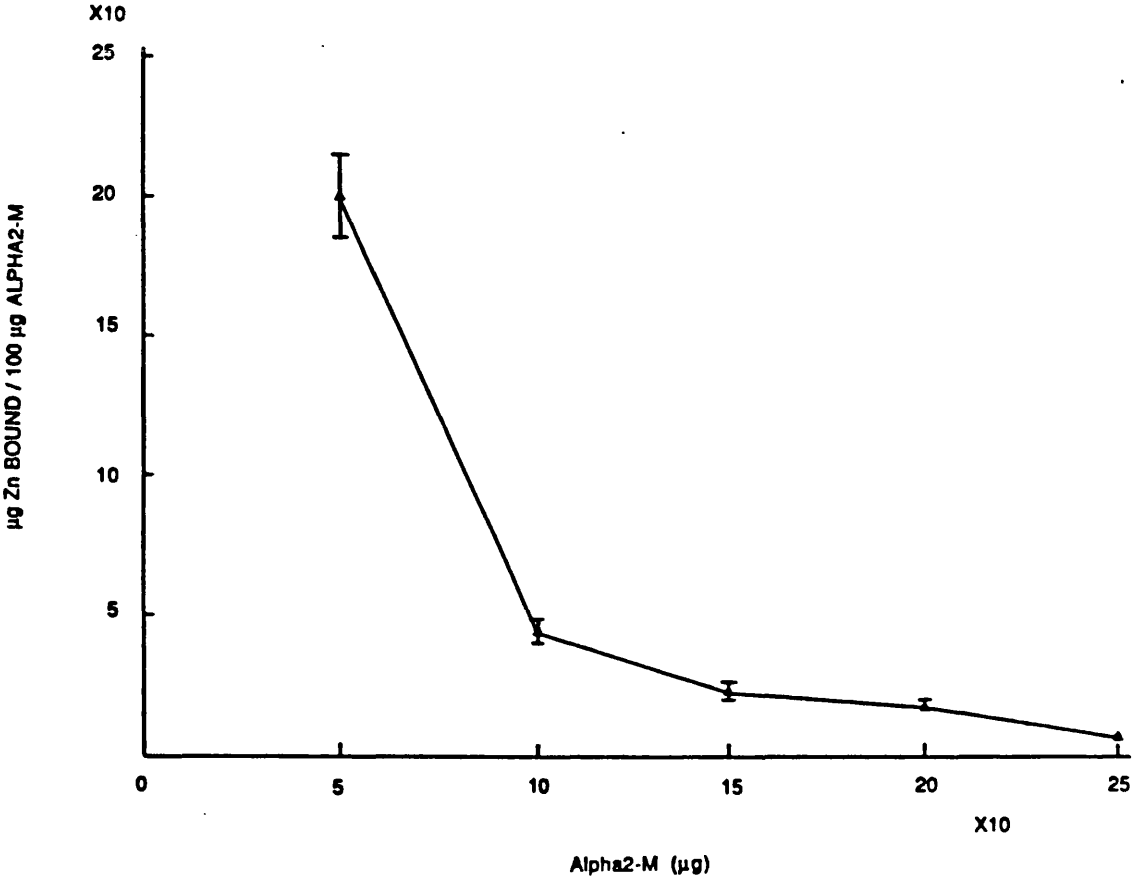


Fig.27 Mean Zn-binding to alpha2-M obtained from sheep plasma incubated with 1500 µg Zn.

DISCUSSION

The most interesting observation from the polyacrylamide gel electrophoresis studies (Tables-I, II & III) was that in all three experiments, maximum concentration of ^{65}Zn was bound to 200 μg of alpha2-M. In Tables-I, II & III the concentrations of 0.0110, 0.0298 and 1.18 μg ^{65}Zn bound respectively to 200 μg alpha2-M. The wide variation in the concentration of Zn bound to 200 μg of alpha2-M could be due to (i) binding of Zn to different binding sites varying in their affinity towards Zn, (ii) differences in the concentration of ^{65}Zn added to alpha2-M. The existence of binding sites with varying affinities is supported by the studies of Adham et al (1977) who reported two classes of binding sites for Zn on alpha2-M, one with greatest affinity for Zn had an apparent stoichiometry (n1in g-atoms/mol of alpha2-M monomer) of 12 and an apparent association constant (K1) of $3.06.10^7$. The second binding site had an n2of 60 and K2 of $1.32.10^5$, with much less affinity. Presence of two classes of Zn-binding sites, a pair of sites per alpha2-M tetramer with highest affinity for Zn and a second class of Zn-binding sites with much lower affinity for Zn were also reported recently by Gettins and Cunningham (1986). Foote and Delves (1984) reported a poor correlationship between the concentration of alpha2-M and its associated zinc was subject to considerable variation. Even with a fourfould difference between the highest and the lowest

points obtained (ratio of alpha2-M to Zn,0.5 to 2.2). Foot and Delves (1984) suggested that the alpha2-M bound Zn fraction is itself subject to metabolic control. This metabolic control could be excercised by dietary concentrations of Zn and Cu and subsequently the liver, which was shown to respond to alterations of Zn and Cu in the diet (Bremner et al,1976).

Despite agreement with Foot and Delves (1984) in observing considerable variations in Zn-binding to alpha2-M, their in vivo observations were in human serum and in this study Zn-binding was studied in vitro. The differences were not only in animal species but also in the technique used.

In Tables-I, II and III g-atoms Zn/mol alpha2-M decreased reflecting the ratio of Zn (conc. of Zn was kept constant) to alpha2-M by weight. As the concentration of alpha2-M increased the concentration of Zn which was maintained at a single level, in each of the experiments was bound to all the available Zn-binding sites.

In Tables-I, II and III initially, more Zn was bound upto 200 µg concentration of alpha2-M and it appeared to reach an equilibrium for the level of Zn available in each of the experiments. Above 200 µg concentration of alpha2-M there was no increase observed in bound Zn compared to 200 µg concentration of alpha2-M. The overall trend of

decrease in Zn-binding was more clearly observed in g-atoms Zn/mol of alpha2-M in Tables-II, III and IV.

Results in this study demonstrated and confirmed that electrophoretic disruption of protein metal interaction did not take place contrary to suggestions made by Song and Adham (1979) and PAGE was used successfully. In this study 5% polyacrylamide gels were used in studying Zn-binding to alpha2-M and the results show similar trend in vertical as well as rod gels. There was no indication of gel percentage used in the work of Song and Adham (1979) and the use of gel with higher percentage could easyly be a factor rather than the technique. However, it is possible to encounter problems particularly when using vertical gel electrophoresis. Despite an advantage, where all samples were subjected to similar electrophoretic conditions, it is possible for samples and radioactivity to overlap. This problem was overcome in this study by leaving a blank sample slot or two, between two samples.

Pratt and Pizzo (1984) showed that on non-denatured polyacrylamide gel electrophoresis of alpha2-M, subunit dissociation was promoted by the presence of metal ions (not all divalent metal ions) of the same oxidation state or orbital structure. Alpha2-M in the presence of 400 μM concentrations of Cu, Hg and Zn showed a marked decrease in the activity of alpha2-M, and this was due to reduction in the stability of intersubunit noncovalent

interactions (Pratt and Pizzo, 1984). However, there is no evidence to suggest that Zn-binding is effected when non-denaturing PAGE was used.

Alpha2-M incubated with low concentration of Zn (Tables-I and II) showed lower Zn-binding than at higher concentration of Zn (Tables-III and IV) and only a part of the total Zn was bound to alpha2-M. These results indicated that alpha2-M has greater Zn-binding capacity. When, the capacity of Zn-binding was more than the utilisable Zn, bound Zn decreased as in Tables-II and III after 200 μg concentration of alpha2-M. It is apparent that irrespective of the concentration of Zn added alpha2-M can only utilise part of the total Zn available.

Maintaining a constant concentration of Zn (1500 μg) and varying the concentration of alpha2-M from 50 to 250μg has shown that alpha2-M has the capacity to bind more zinc and the available zinc was shared by the binding sites available and increasing the alpha2-M concentration has decreased the concentration of Zn bound to alpha2-M. Whether, a similar response to increased Zn levels against a constant concentration of alpha2-M was possible will be investigated in the next experiment.

Results in Table-IV showed that when more Zn was available alpha2-M appeared to have the capacity to bind

more Zn and only a proportion of the available Zn could be utilised.

EXPERIMENT-3

INFLUENCE OF VARIABLE AMOUNTS OF Zn ON Zn-BINDING TO ALPHA2-M

INTRODUCTION

In the earlier experiments it was shown that the capacity of alpha2-M to bind Zn depends upon the level of Zn with which it is associated. It was also shown that only a proportion of the available Zn is bound to increasing concentrations of alpha2-M.

Further study of Zn-binding properties of alpha2-M was carried out to examine whether a relationship exists between variable Zn concentration and alpha2-M.

RESULTS

Results presented in Table-V and Fig.28 showed a linear relationship between the amount of Zn added to that of alpha2-M, which was kept constant at 100 μg. As the Zn concentration increased from 150 μg Zn to 3000 μg Zn, the uptake of Zn increased from 14.45 to 293.41 mean μg Zn bound/100 μg of alpha2-M. Twenty times more Zn was bound to alpha2-M during an increase in added Zn from 150 to 3000 μg Zn (20 times increase). These results are highly significant ($P<0.001$).

The g-atoms Zn/mol of alpha2-M increased from $16.03.10^2$ to $325.44.10^2$ consistent with the increase in Zn concentration.

Table- V

Zn- binding to 100 µg of alpha2-M on varying incubated Zn from 150- 3000 µg.

Zn added (µg)	No.	µg Zn Bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/ mol alpha2-M
150	5	14.45 ***	± 0.03	$16.03.10^{2}$
300	5	28.06 ***	+ 1.03	$31.12.10^{2}$
450	5	45.21 ***	+ 1.47	$50.14.10^{2}$
750	5	74.55 ***	± 0.61	$82.69.10^{2}$
1500	5	150.90 ***	± 1.84	$167.37.10^{2}$
3000	5	293.41 ***	± 4.38	$325.44.10^{2}$

*** $P < 0.001$

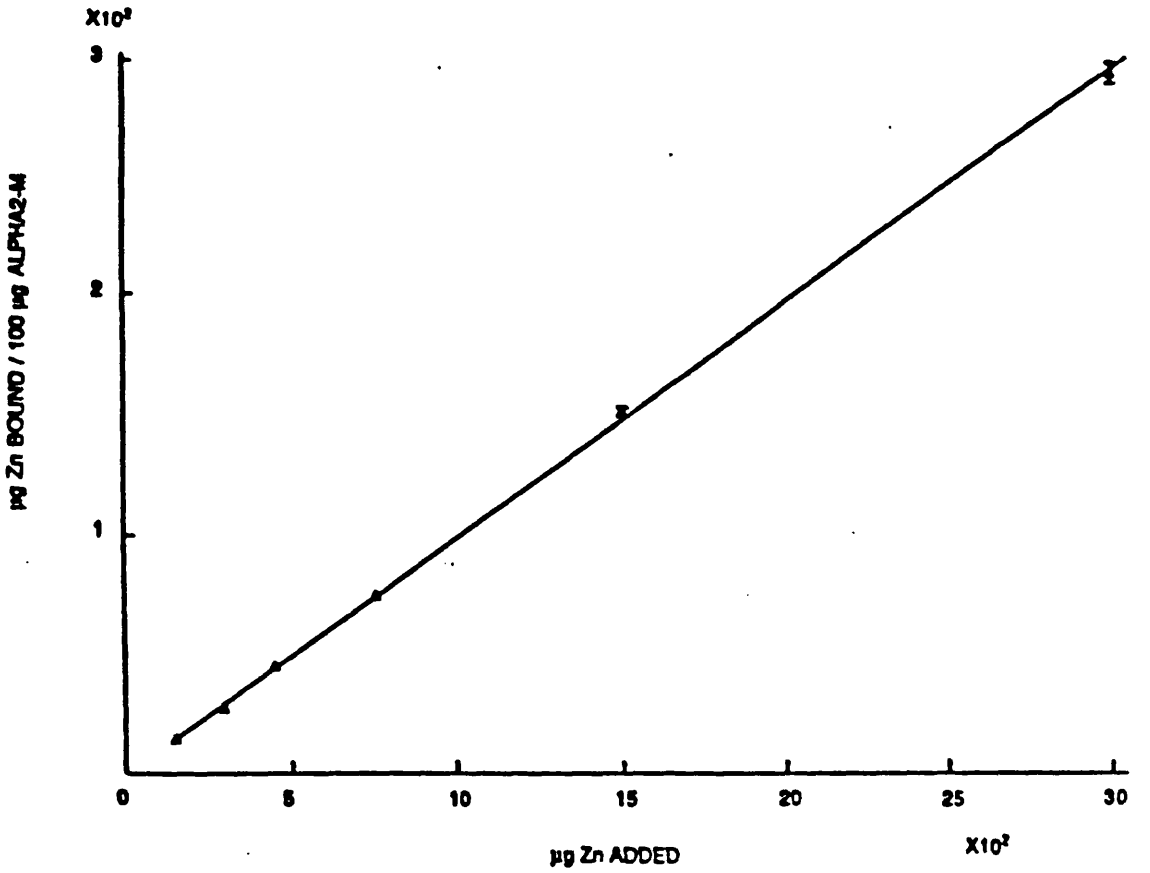


Fig.28 Mean Zn-binding to 100 µg of alpha2-M obtained from sheep plasma.on varying incubated Zn from 150 to 3000 µg.

DISCUSSION

Increased Zn concentration in the incubation mixture with alpha2-M has shown a linear relation ship (Table-V, Fig.28) when the concentration of alpha2-M was constant (100 μg). These results confirmed the earlier observations that alpha2-M has the capacity to bind Zn possibly far in excess of the concentrations reported.

The studies of Adham et al (1977) showed that saturation of Zn-binding site 1 with Zn did not have any effect. However,at 20 g-atoms Zn/mol alpha2-M, 25% of trypsin binding activity was lost and at 70 g-atoms Zn/mol alpha2-M practically lost all trypsin binding activity. Alpha2-M dialysed against EDTA buffer did not restore the activity. On the basis of these observations they concluded, that the occupation of the second binding site by Zn, initiated an irreversible conformational changes in the molcule interfering with trypsin binding activity. They suggested that each mol of alpha2-M may bind upto 70 g-atoms Zn before all of these sites are saturated. Gonias et al (1984) are in agreement with this.

Gonias et al (1984) showed that incubation of human alpha2-M with 5 μM to 1.0 mM $ZnCl_2$ solutions did not precipitate. However, alpha2-M-trypsin and alpha2-M-methylamine precipitated (80%) in concentrations over 0.2 mM $ZnCl_2$. They showed that these precipitated proteins contained fast forms of alpha2-M and involved metal ion-induced conformational changes in the alpha2-M as reported by Adham et al (1977). No

precipitation of alpha2-M was observed in this study using upto 3000 μg Zn in vitro (230 mM), which suggests absence of fast-form of alpha2-M. These studies indicate the possibility of higher Zn-binding values and results in this study confirmed these high values subject to the availability of Zn.

In their dialysis studies Adham et al (1977) showed 50% higher Zn concentration in human alpha2-M than that of the albumin based on the weight of protein . In terms of g-atoms Zn/mol of protein,the Zn content of alpha2-M was 20 times higher than that of albumin (Adham et al,1977). In subsequent experiments sheep alpha2-M and albumin will be examined for their binding affinities.

Evidence from these in vitro experiments show that the alpha2-M has a greater Zn-binding capacity than has been previously accounted for from in vivo observations. Pratt and Pizzo (1984) suggested that the 30-40% of zinc bound to human alpha2-M claimed by Parisi and Vallee (1970) is more likely to reflect a maximum proportion of zinc bound to alpha2-M, which is not normallyfound in normal in vivo states.

The results in Table-V showed the g-atoms Zn/mol of alpha2-M far in excess of 70 g-atoms Zn for human alpha2-M (Adham et al, 1977), which strongly suggests the possibility of conformational changes occuring in sheep alpha2-M in response to higher Zn concentrations, as oberved by Adham et al (1977) in human plasma alpha2-M. In sheep the possibility of higher Zn concentrations, particularly through ingestion of diets or

dietary imbalances created by Cu antagonism, though remote, should be considered. In the subsequent studies experimental evidence will be presented in support of the hypothesis that dietary Cu concentration influences Zn-binding to alpha2-M in sheep plasma and is dependent on the ratio of dietary concentrations of Cu and Zn.

EXPERIMENT-4

AN IN VITRO STUDY OF THE COMPETITIVE BINDING BETWEEN COPPER AND ZINC FOR ALPHA 2-MACROGLOBULIN

INTRODUCTION

In this experiment the influence of Cu on Zn-binding to alpha2-M was examined and was related to the concentration of Cu and Zn in the diet.

Two techniques of study were used for this, PAGE and gel filtration on Sephadex G-200. Details of these methods are described in Section-II.

RESULTS

Results are presented in Table-VI and Fig.29. When the effect of Cu upon Zn-binding was studied using the gel filtration binding method (Section-II, 8b) there was a significant ($P<0.001$) decrease in Zn-binding to alpha2-M at level-I (150 μg Zn), when the Cu concentration was increased from 5 to 25 μg Cu. Increasing the concentration from 5 to 10 μg Cu does demonstrate a significant ($P<0.001$) increase in Zn-binding. Higher concentration of Cu, however, has decreased the affinity of Zn to alpha2-M, resulting in 55.8% reduction with 25 μg Cu, compared to the Zn-binding at 5 μg Cu level. This effect was also seen with level-II Zn (1500 μg Zn) and 5

Table- VI

Effect of in vitro Cu (5-25 µg) and in vitro Zn (150 µg) on Zn-binding to alpha2-M in sheep plasma.

Cu added (µg)	No. of assays	µg Zn Bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/ mol alpha2-M
5	5	18.37 ***	± 1.50	$20.37.10^{2}$
10	5	19.77 ***	± 0.77	$21.93.10^{2}$
15	5	14.76 ***	± 1.12	$16.37.10^{2}$
20	5	12.16 ***	± 0.70	$13.49.10^{2}$
25	5	8.11 ***	± 0.52	$8.99.10^{2}$

*** $P < 0.001$

Table-VII

Effect of in vitro Cu (5-25 µg) and in vitro Zn (1500µg) on Zn-binding to alpha2-M in sheep plasma.

Cu added (µg)	No. of assays	µg Zn Bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/ mol alpha2-M
5	5	170.55 ***	± 0.84	$198.16.10^{2}$
10	5	158.98 ***	± 3.19	$176.33.10^{2}$
15	5	113.47 ***	± 4.96	$125.85.10^{2}$
20	5	126.98 ***	± 2.82	$140.84.10^{2}$
25	5	98.98 ***	± 2.24	$109.78.10^{2}$

*** $P < 0.001$

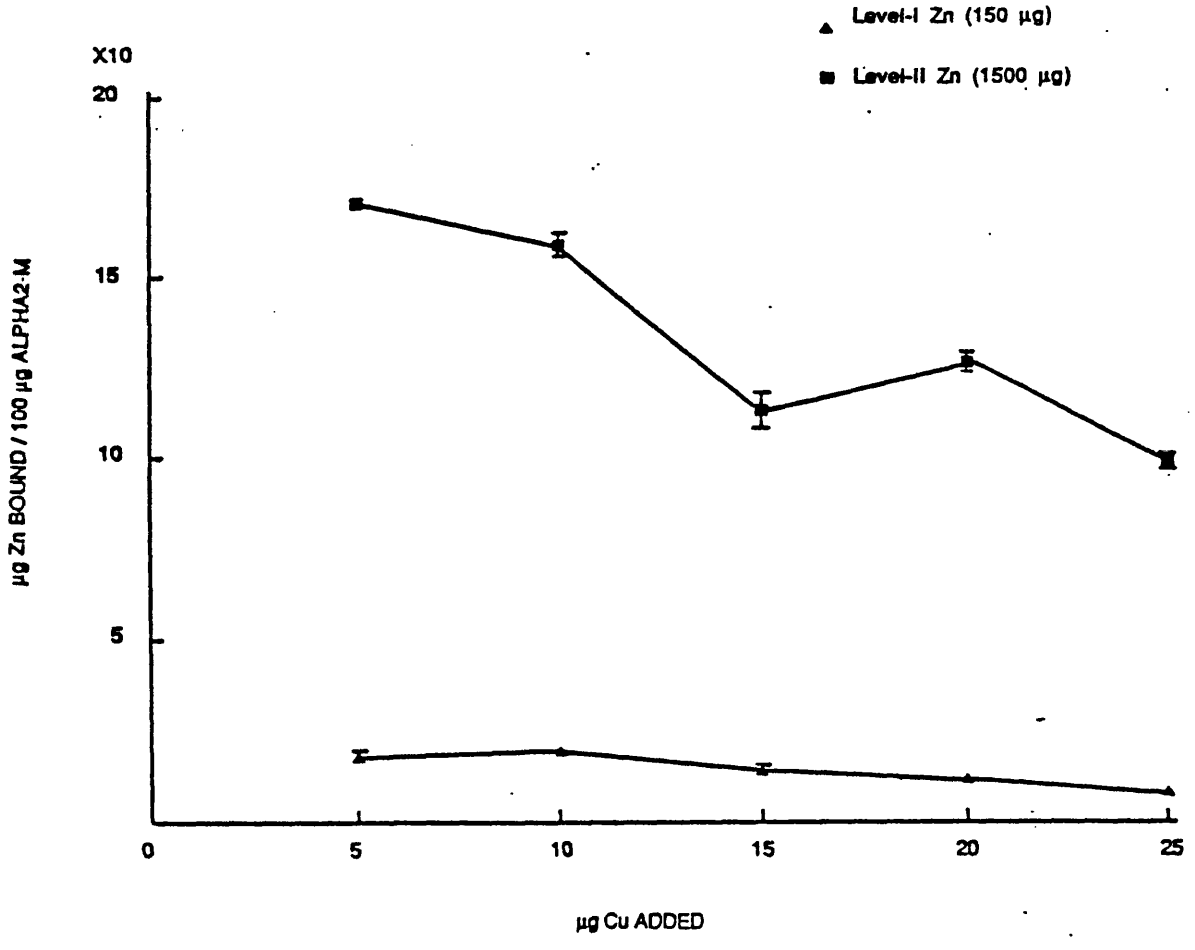


Fig.29 The effect of in vitro Cu (5-25 ug) and two levels of Zn (150 and 1500 ug) on Zn-binding to alpha2-M obtained from sheep plasma using gel filtration method.

to 25 μg Cu (Table-VII, Fig.29). There was a 42% decrease in Zn-binding. A significant ($P<0.001$) decrease was observed at each Cu level compared to binding in the presence of 5 μg Cu.

In contrast there was no clear relationship between Cu and Zn-binding when 5% PAGE was used. There was a trend to decreased Zn-binding with concentrations of 10-30 μg Cu but this was reversed with 50 μg Cu. This effect of Cu was less marked at 72 hours when the binding of Zn was with the exception of the 30 μg sample, similar.

Results presented in Table-VIII and Fig.30 show a similar trend in percent ^{65}Zn bound, μg ^{65}Zn bound and g-atoms Zn/mol of alpha2-M.

Although the following results are those of single values for increases in copper concentration,they do however reflect a trend of importance which needs further confirmation.

When the influence of each Cu concentration was assessed different trends emerged. The effectiveness of Cu level in the incubation mixture was assessed by increase and decrease in μg ^{65}Zn bound and g-atoms Zn/mol of alpha2-M in all.

The incubation of alpha2-M with 10 μg Cu was least

TABLE- VIII

Effect of in vitro Cu ranging from 10 to 50 µg and Zn, incubated for 24 to 216 hours on Zn-binding to alpha2-M in sheep plasma.

Hours of Incubation

Cu conc. (µg)		24	72	108	216
10	%65Zn bound	5.4	8.7	4.3	3.6
	µg 65Zn bound	0.0110	0.0174	0.0086	0.0073
	g-atoms Zn/mol alpha2-M	1.22	1.93	0.95	0.81
20	%65Zn bound	3.4	7.6	2.1	2.6
	µg 65Zn bound	0.0068	0.0152	0.0041	0.0052
	g-atoms Zn/mol alpha2-M	0.75	1.68	0.45	0.58
30	%65Zn bound	2.6	3.7	2.7	4.5
	µg 65Zn bound	0.0053	0.0073	0.0054	0.0090
	g-atoms Zn/mol alpha2-M	0.59	0.81	0.60	1.00
40	%65Zn bound	2.96	7.57	2.25	7.2
	µg 65Zn bound	0.0059	0.0151	0.0045	0.0144
	g-atoms Zn/mol alpha2-M	0.65	1.67	0.50	1.60
50	%65Zn bound	7.0	8.4	3.94	3.8
	µg 65Zn bound	0.0141	0.0168	0.0079	0.0076
	g-atoms Zn/mol alpha2-M	1.56	1.86	0.87	0.84

65Zn added = 0.1 µCi = 0.2 µg Zn

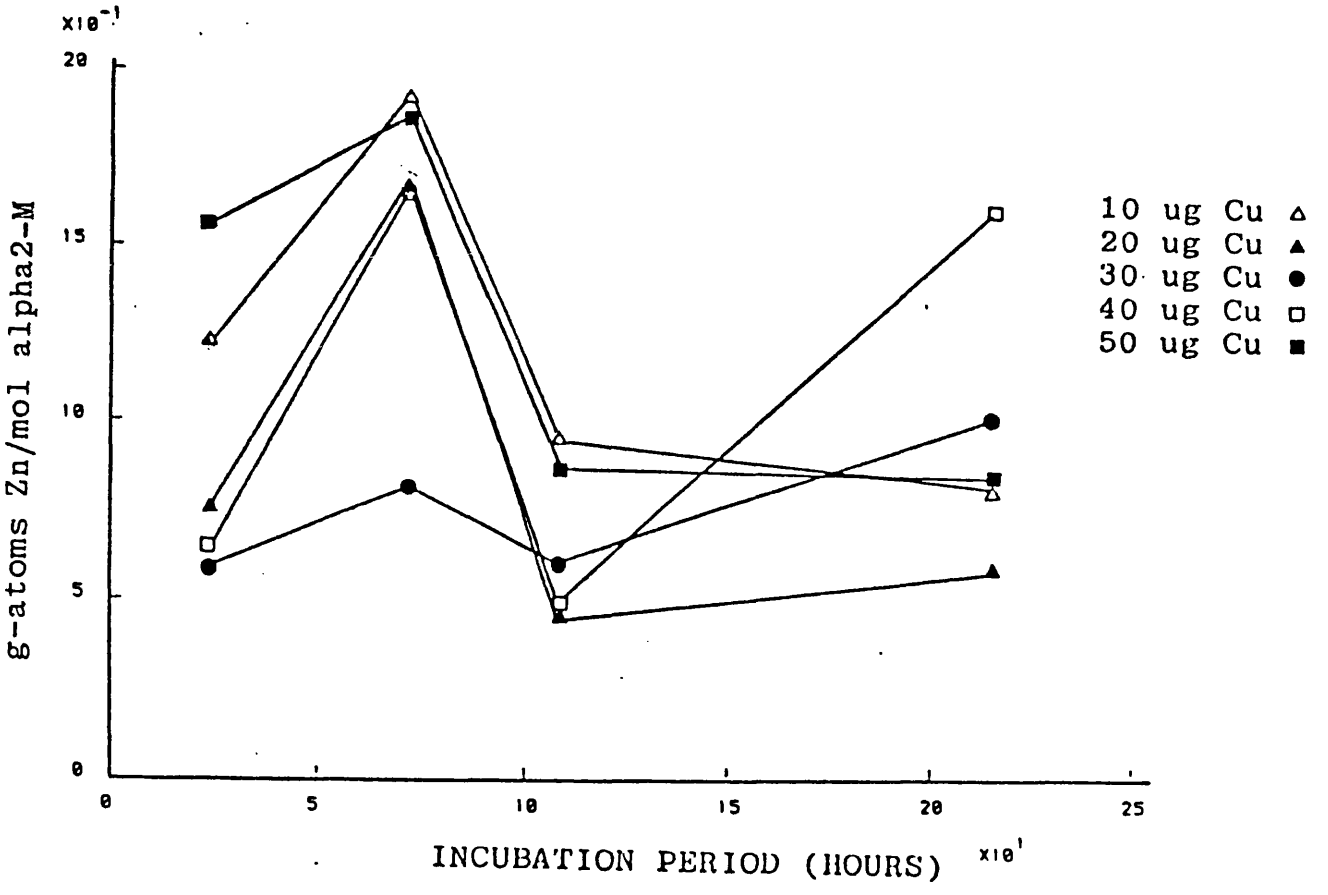


Fig.30 Effect of Cu (10-50 ug) and incubation period (24-216 hours) on Zn-binding to alpha2-M using 5 per cent PAGE (vertical gel).

effective at 72 hours and most effective at 216 hours (Table-VIII, Fig.30).

Concentration 20 μg Cu with alpha2-M was least effective after 72 hours and most effective after 108 hours (Table-VIII, Fig.30).

Incubation of alpha2-M with 30 μg Cu was least effective after 216 hours and was most effective after 24 hours (Table-VIII, Fig.30).

Alpha2-M incubated with 40 μg Cu was most effective after 108 hours and least effective after 72 hours (Table-VIII, Figs.30).

Cu concentration of 50 μg was most effective after 216 hours and least effective after 72 hours (Table-VIII, Figs.30).

DISCUSSION

Two experiments have essentially been carried out under the title for Expt.4; these differed in technique and method in the study of the competition between Cu and Zn for alpha2-M binding.

The differences between these two were: 1.Cu was presented to alpha2-M before the two levels (150 μg and 1500 μg Zn) of Zn were added; gel filtration was then used.
2.Cu was presented with Zn simultaneously to alpha2-M; PAGE was then used.

The essential result from these approaches showed evidence that whilst Cu (*in vitro*) can compete with Zn for binding to alpha2-M, the time of exposure (or incubation) of both these elements to alpha2-M was important. This became obvious when the *in vitro* Cu was incubated alone for 24 hours with alpha2-M because the first experiment using the gel filtration technique showed less alpha2-M bound Zn than when Cu with Zn were incubated together for periods between 24-216 hours with alpha2-M when the second experiment using the PAGE technique showed more Zn bound to alpha2-M.

The first experiment:

1. EFFECT OF IN VITRO Cu ON IN VITRO Zn-BINDING TO ALPHA2-M IN SHEEP PLASMA

Results in Tables-VI & VII (Fig.29) showed significantly ($P<0.001$) decreased Zn-binding to alpha2-M after 24 hours incubation with Cu concentrations between 5 to 25 μg. This result was shown at both levels of *in vitro* Zn, viz. 150 and 1500 μg Zn (Fig.29). This supported the results during 24-216 hours in Table-VIII using the PAGE technique. Thus demonstrating clear evidence of *in vitro* Cu decreasing Zn-binding to alpha2-M.

^{65}Zn was added 10-30 minutes before application on gel filtration column to assay the bound Zn. Thus the alpha2-M incubated Cu was probably occupying a weaker class of Zn-binding site on alpha2-M prior to exposure to 150 μg Zn and 1500 μg Zn as shown in Tables-VI & VII, Fig.29. Hence, despite increase in Zn-binding at 10 μg Cu concentration the over all trend showed a decrease in Zn-binding to alpha2-M at both levels of Zn.

Adham et al (1977) further supported by Pratt and Pizzo (1984), Gettins and Cunningham (1986) classified Zn-binding sites on human alpha2-M into two, sites 1 and 2, strong Zn-binding and weaker Zn-binding respectively. It would appear from these results assuming sheep

alpha2-M have similar binding properties that the Cu competing and partially replacing bound Zn has taken place on sites 2 (Adham et al, 1977) on alpha2-M.

The second experiment:

2 COMBINED INFLUENCE OF IN VITRO Cu, Zn AND INCUBATION PERIOD ON Zn-BINDING TO ALPHA2-M

Results of Zn-binding to alpha2-M (Table-VIII, Fig.30) showed two clear phases. During the first phase, upto 72 hours, alpha2-M bound Zn increased with 10-50 μg of *in vitro* Cu. During the second phase, from 72 to 216 hours, the alpha2-M bound Zn decreased at all levels of *in vitro* Cu (10, 20, 40 and 50 μg), except at 30 μg Cu level. The rise in Zn-binding during the first phase was probably due to the availability of Zn-binding sites which were demonstrating greater affinity, as in Adham's et al (1977) sites 1, for Zn than Cu. Support for this view is available from the studies of Pratt and Pizzo (1984). They showed that under electrophoretic conditions employed in their study, some subunit dissociation took place. Thus Zn could have become bound to the available Zn-binding sites on such subunits.

The results in the current study demonstrated and confirmed that Zn-binding was influenced by Cu and the differences in the concentrations of Zn, Cu and the

period of incubation appeared to be responsible for the decrease in Zn-binding to alpha2-M. The differences observed could be due to competition for the sites between Cu and Zn at both high and low affinity (strong and weaker) sites.

During an incubation period of 216 hours the variation in Zn-binding reduced by over 40% for most of the incubation times with the different Cu concentrations used. Thus possibly demonstrating the importance of adequate incubation time to allow this decreased effect when both Cu and Zn are in the incubation medium. The influence of Cu in this competition is further supported on comparison of the results in Table-VIII with Table-V (Expt.3) where the results were obtained in the absence of Cu.

It is possible that there may be stereochemical factors, shown by Banaszak et al (1965) in human myoglobin with two specific binding sites, one for Cu and another for Zn, similar factors may be responsible in sheep plasma alpha2-M which can influence Zn-binding in preference to Cu during the first phase. Such stereochemical factors could influence binding affinities of Cu and Zn. Though the situation is different in sheep alpha2-M with only Zn-binding sites with high and low affinities for Zn, these stereochemical factors need further investigation in sheep plasma alpha2-M.

Zn-binding to alpha2-M during the first phase could be under homeostatic control. However, the affinity for Zn was not apparent during second phase and thus no evidence of homeostatic control; this was probably due to replacement of Zn by Cu at weaker Zn-binding sites.

It is equally worth noting that the concentration of ^{65}Zn added (equivalent to 0.2 or 2.0 μg Zn) to the mixture containing alpha2-M and Cu was far less than Cu concentration (10 to 50 μg) to exhibit sufficient influence at the weaker binding sites. Excess Cu in the incubation mixture may be responsible for the breakdown of homeostatic mechanism.

During the second phase, the longer incubation period combined with the effect of Cu and electrophoresis might have caused dissociation of alpha2-M. This would make the weaker Zn-binding sites available for excess Cu in the incubation mixture and unavailable for Zn by giving preference to Cu due to greater affinity of this element to bind to proteins (Williams, 1984; Bremner and Marshall, 1974).

EXPERIMENT-5

EFFECT OF DIETARY COPPER DEFICIENCY ON ZINC BINDING TO ALPHA 2-MACROGLOBULIN

INTRODUCTION

In vitro experiments have supported a role of Cu in modifying the Zn-binding of alpha2-M. In this study a Cu-deficient diet was fed to sheep to investigate the *in vivo* influence of Cu-deficiency on *in vitro* Zn-binding to alpha2-M.

RESULTS

Mean plasma Zn concentrations (Table-IX, Fig.31) in Cu-deficient sheep decreased significantly ($P<0.001$) and remained low for a period of 9 weeks compared to the mean concentrations at the begining of the experiment. Despite providing a diet supplemented with 5 mg Cu/kg diet after week 7 the mean Zn concentrations remained lower. During the 7 weeks on a Cu-deficient diet and during weeks 8 and 9 on Cu-supplemented diet, the mean Zn concentrations of plasma were variable.

The mean Cu concentrations (Table-IX, Fig.31) in Cu-deficient sheep plasma remained low even after supplementing the diet with 5 mg Cu/kg diet.

Table- IX

Mean Zn and Cu concentrations of Cu-deficient sheep plasma.

		µg Zn/ml plasma		µg Cu/ml plasma	
Week	No	Mean	S.E.M.	Mean	S.E.M.
0	5	1.14 ***	± 0.09	1.19 **	± 0.19
1	5	0.90 ***	± 0.07	0.74***	± 0.09
2	5	0.85 ***	± 0.05	0.79***	± 0.08
3	5	0.94 ***	± 0.08	0.81***	± 0.06
4	5	0.87 ***	± 0.07	0.71***	± 0.05
5	5	0.85 ***	± 0.06	0.74***	± 0.05
6	5	0.98 ***	± 0.09	0.83***	± 0.08
7	5	1.12***	± 0.04	0.88***	± 0.07
8	5	0.76***	± 0.05	0.85 ***	± 0.09
9	5	0.88***	± 0.02	0.82 ***	± 0.05

** $P < 0.01$ *** $P < 0.001$

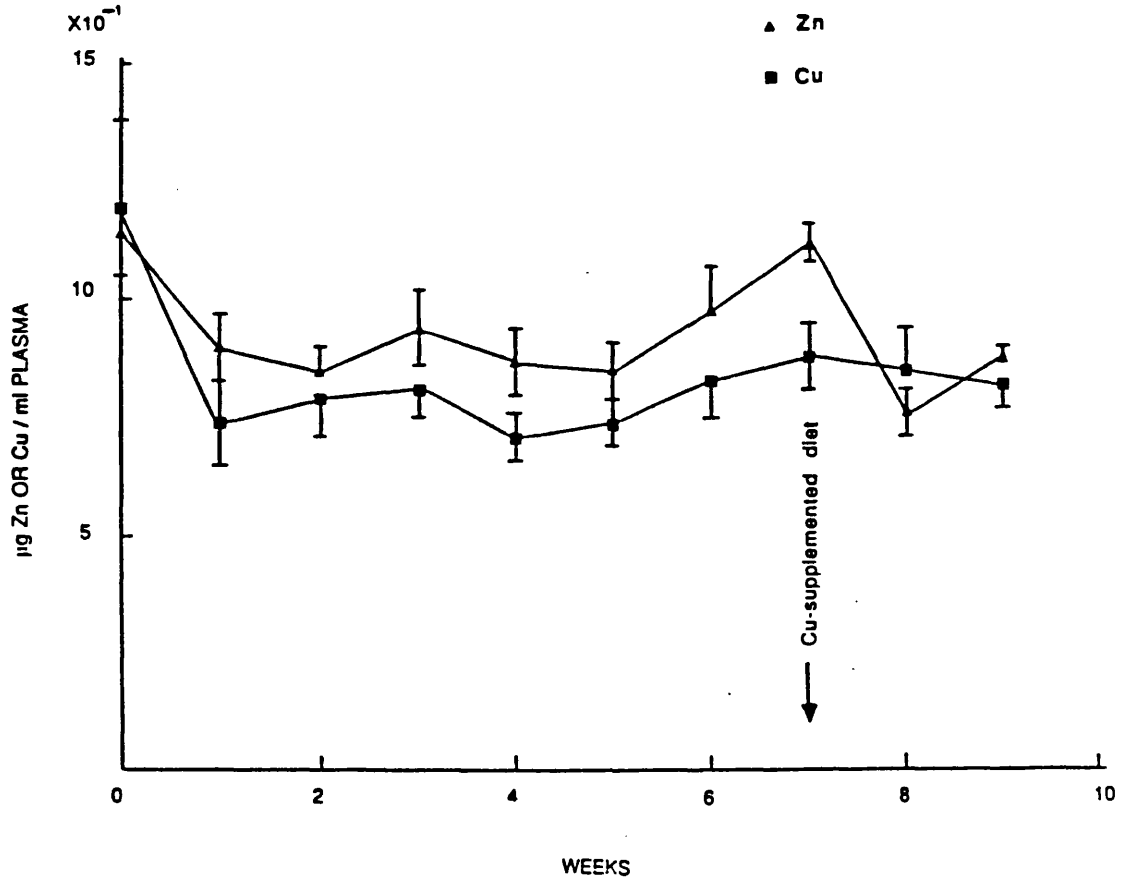


Fig.31 Mean Zn and Cu concentrations of Cu-deficient sheep plasma.

Results of Zn-binding to alpha2-M in Cu-deficient sheep plasma are presented in Tables-X & XI and Fig.32. It is interesting to observe a pattern of variation of Zn-binding to alpha2-M. This variation in the Zn-binding seems to have a cyclic effect every 2 to 3 weeks.

In vitro Zn-binding to alpha2-M was studied at two levels of Zn incubation, level-I, 150 μg Zn;and level-II, 1500 μg Zn.

In Cu-deficient sheep alpha2-M incubated with level-I Zn, first showed significantly ($P<0.001$) decreased Zn-binding after one week and then increased Zn-binding to alpha2-M from 40.10 to 67.26 μg Zn/100 μg alpha2-M during the first four weeks, which was equivalent to $44.48 \cdot 10^2$ and $74.60 \cdot 10^2$ respectively. After 3 weeks on Cu-deficient diet Zn-binding to alpha2-M was at maximum, 112.27 μg Zn/100 μg alpha2-M ($124.52 \cdot 10^2$ g-atoms Zn/mol alpha2-M), 2.8 times or 180% increase when compared to Zn-binding at the begining of the experiment. Despite observing 1.7 times Zn-binding to alpha2-M (67.7%) after 4 weeks, by week 5 Zn-binding to alpha2-M decreased and was nearly 58% lower than at the start of the experiment. When Cu-supplemented diet (5 mg Cu/kg diet) was fed after week 7 Zn-binding to alpha2-M showed a trend of increased Zn-binding in weeks 8 and 9, which were 55.02 and 51.85 μg Zn/100 μg alpha2-M respectively.

Table- X

Zn- binding to alpha2-M obtained from Cu-deficient sheep plasma incubated with 150 µg Zn (level-I).

Week	No.	µg Zn bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/ mole alpha2-M
0	5	40.10***	± 1.66	$44.48.10^2$
1	5	34.99***	± 1.74	$38.81.10^2$
2	5	69.95***	± 2.05	$77.58.10^2$
3	5	112.27***	± 3.23	$124.52.10^2$
4	5	67.26***	± 3.75	$74.60.10^2$
5	5	18.11***	± 1.31	$20.09.10^2$
6	5	44.02***	± 2.11	$48.82.10^2$
7	5	10.05***	± 1.22	$11.15.10^2$
8	5	55.02***	± 2.23	$61.02.10^2$
9	5	51.85***	± 3.20	$57.51.10^2$

*** $P < 0.001$

Table- XI

Zn-binding to alpha2-M obtained from Cu-deficient sheep plasma incubated with 1500 µg Zn (Level-I).

Week	No of assays	µg Zn bound/ 100 µg alpha2-M		g-atoms Zn/ mol alpha2-M
		Mean	S.E.M.	
0	5	349.2 ***	± 11.90	$38.73.10^3$
1	5	456.5 ***	± 19.10	$50.63.10^3$
2	5	974.7 ***	± 26.40	$108.11.10^3$
3	5	3249.9 ***	± 72.10	$360.46.10^3$
4	5	827.6 ***	± 30.30	$91.79.10^3$
5	5	710.0 ***	± 10.40	$78.75.10^3$
6	5	1882.5 ***	± 39.40	$208.80.10^3$
7	5	201.8 ***	± 16.10	$22.38.10^3$
8	5	1118.6 ***	± 16.20	$124.07.10^3$
9	5	378.1 ***	± 22.50	$41.94.10^3$

*** $P < 0.001$

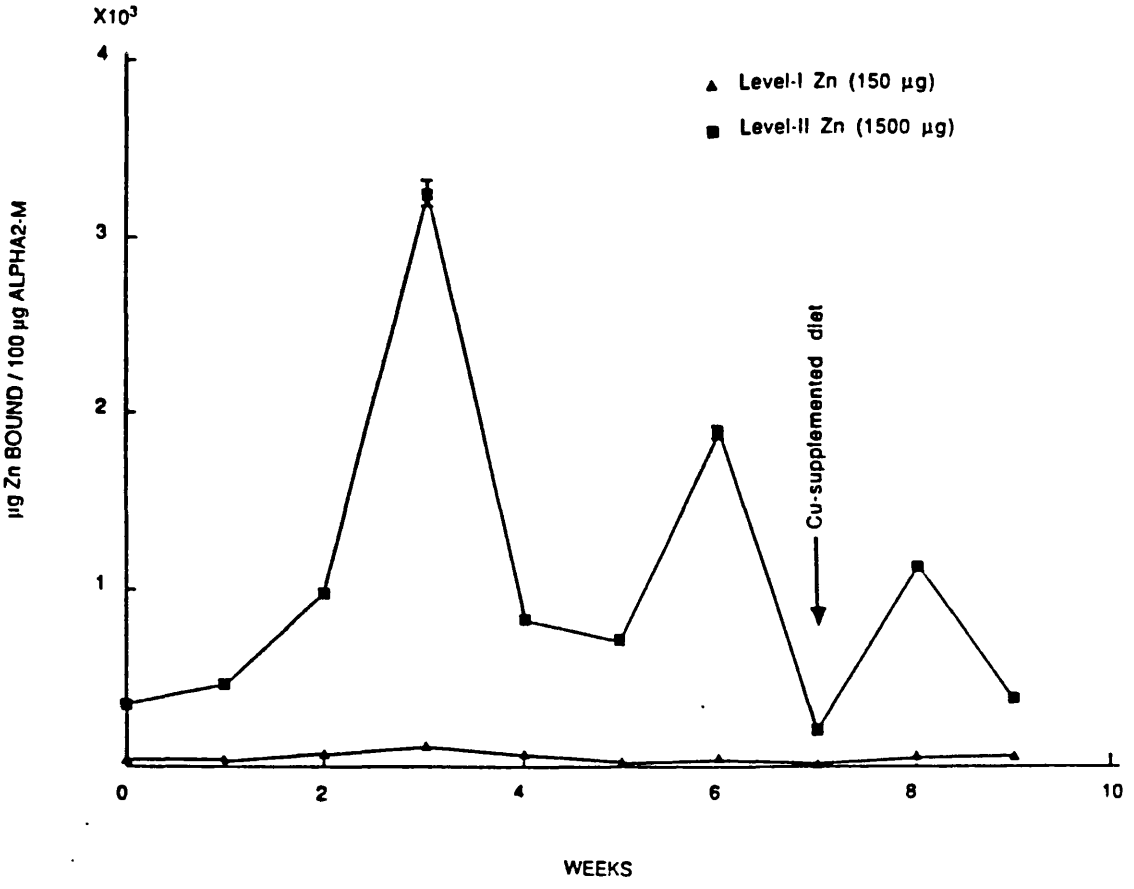


Fig.32 Zn-binding to alpha2-M obtained from Cu-deficient sheep plasma incubated with two levels of Zn (150 and 1500 µg).

In vitro Zn-binding to alpha2-M in Cu-deficient sheep plasma incubated with level-II Zn (1500 μg) significantly ($P<0.001$) increased upto 6 weeks (Table-XI, Fig.32). After 6 weeks 439% (5.4 times) increase in vitro binding of Zn to alpha2-M was observed. At one point after week 3 maximum binding was 3249.9 μg Zn/100 μg alpha2-M (equivalent to 360.46 $.10^3$ g-atoms Zn/mol alpha2-M, an increase of 830.7%). Despite the Zn-binding to alpha2-M decreasing in weeks 5 and 6 increased Zn-binding to alpha2-M was maintained at a higher level during weeks 5 and 6. Lowest Zn-binding to alpha2-M was after week 7, which was 201.8 μg Zn/100 μg alpha2-M, 42.2% less than at the begining of the experiment. Feeding Cu supplemented diet (5 mg Cu/kg diet) maintained Zn-binding at higher level than at the begining, during weeks 8 and 9.

Zn concentration of Pool-I and Pool-II (Table-XII, Fig.33) showed reduction of Pool-II Zn as a consequence of this the ratio (ratio of Pool-I Zn compared to Pool-II) of Zn in Pool-I increased from week 3 and remained higher than Pool-II Zn. Though Zn concentration in both pools decreased Pool-II demonstrated greater reduction. In Pool-I after 7 weeks on Cu-deficient diet, sheep plasma alpha2-M Pool Zn (Pool-I) concentration increased by only 2%. On the other hand 34.5% decrease in Zn concentration of albumin Pool was observed. After

Table- XII

Zn concentration of alpha2-M pool (Pool-I) and albumin pool (Pool- II) in Cu- deficient sheep plasma.

Week	Pool-I	Pool-II	Ratio of Zn I:II
0	0.600	0.800	0.75:1
1	0.405	0.516	0.78:1
2	0.360	0.485	0.74:1
3	0.498	0.460	1.08:1
4	0.482	0.380	1.27:1
5	0.440	0.425	1.03:1
6	0.546	0.430	1.27:1
7	0.612	0.524	1.17:1
8	0.408	0.372	1.10:1
9	0.502	0.394	1.27:1

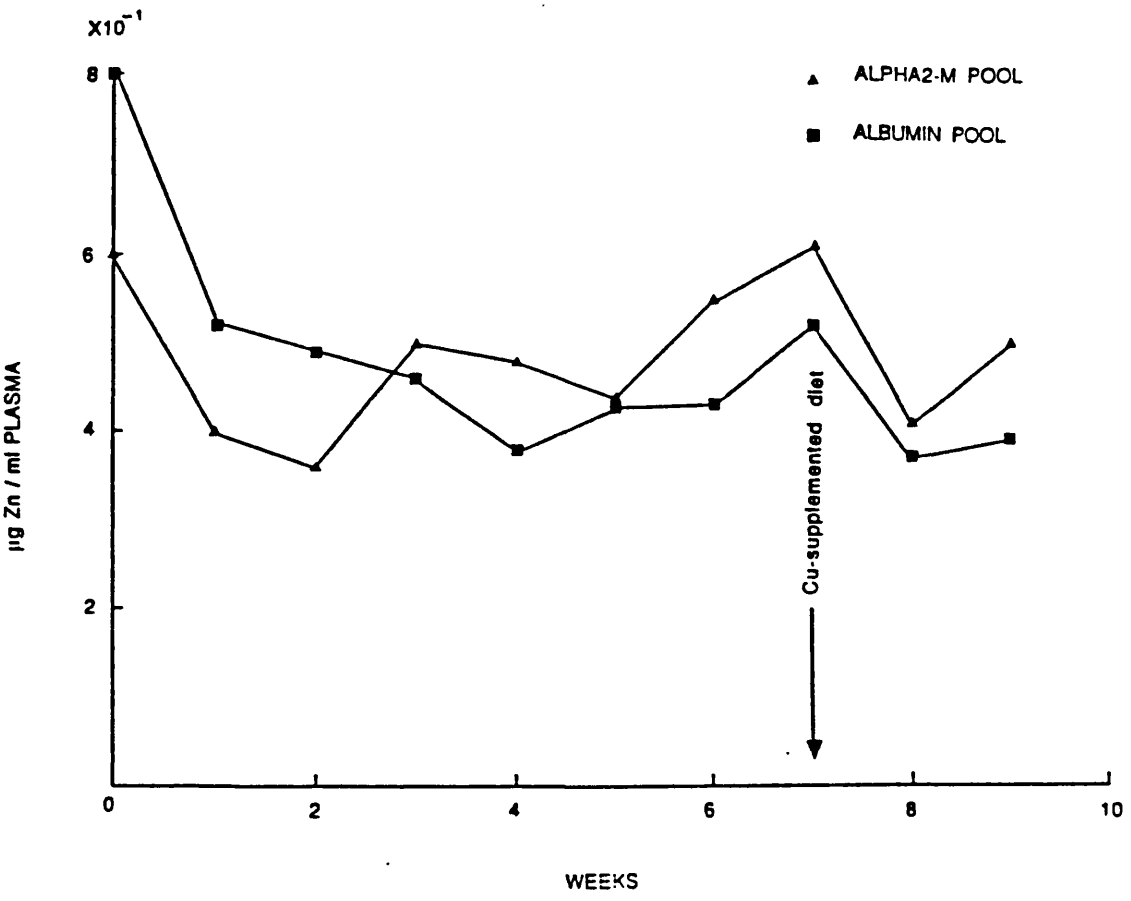


Fig.33 Zn concentration of alpha2-M pool (Pool-I) and albumin pool (Pool-II) in Cu-deficient sheep plasma.

feeding Cu-supplemented diet (5 mg Cu/kg diet) there was no increase in either Pool-I or Pool-II Zn concentration (Table-XII).

The Zn concentration of purified alpha2-M (Table-XIII, Fig.34) obtained from Cu-deficient sheep plasma showed reduction of Zn after one week and showed inreased Zn concentration of alpha2-M upto week 3. After which time showed lower Zn concentration than at the begining of the experiment. Dietary supplementation of Cu appeared to inrease Zn concentration during week 8.

There was no Cu present in purified alpha2-M in Cu-deficient sheep plasma.

The results of dialysis of alpha2-M and albumin mixed with 2000 μg Zn (Table-XIV, Fig.35) showed 4.55% Zn remained with alpha2-M and 2.37% Zn with albumin after 24 hours. After 24 hours dialysis alpha2-M showed 50% higher bound Zn than that of albumin based on weight of the proteins and 350% higher than albumin after 7 days. In terms of g-atoms Zn/mol of protein, 50 times more Zn was bound to alpha2-M than albumin after 7 days.

Table- XIII

Zn and Cu concentrations of alpha2-M obtained from Cu- deficient sheep plasma.

Week	mg Zn/ g alpha2-M	mg Cu/g alpha2-M
0	1.42	-
1	0.54	-
2	3.12	-
3	10.75	-
4	1.29	-
5	0.45	-
6	0.59	-
7	0.18	-
8	1.50	-
9	0.57	-

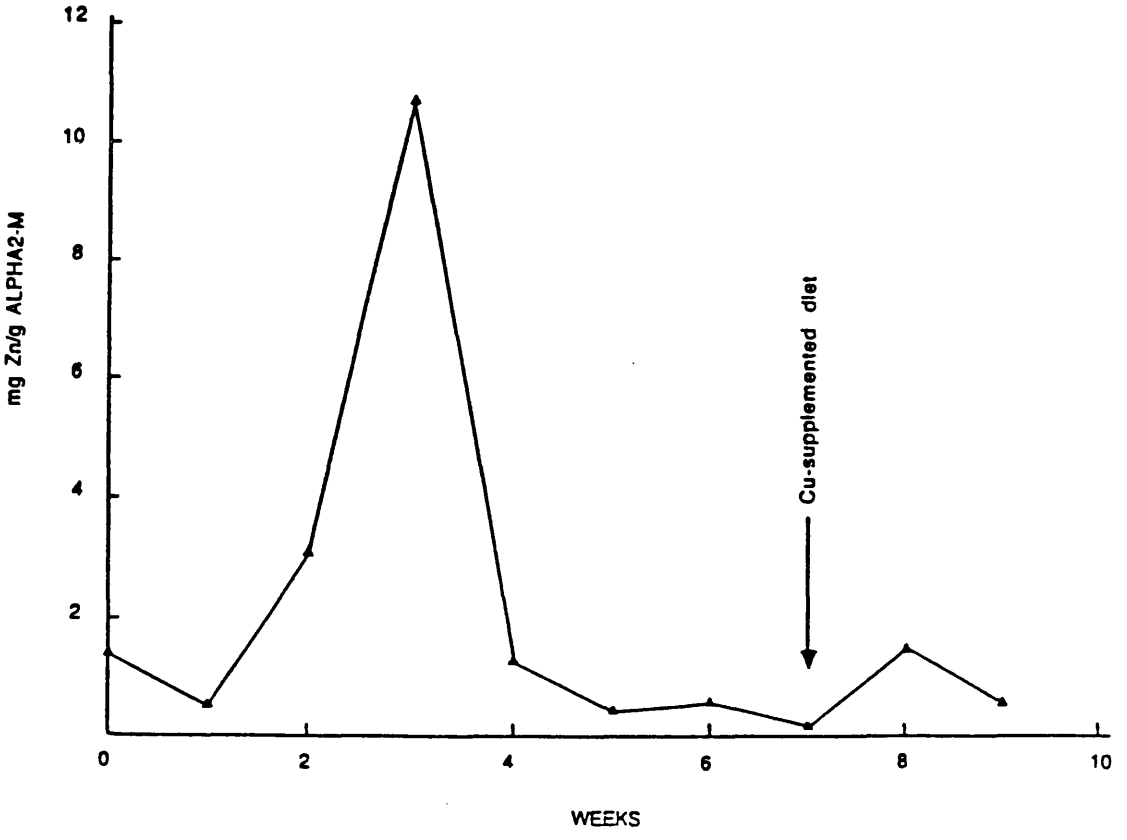


Fig.34 Zn concentration of alpha2-M obtained from Cu-deficient sheep plasma.

Table- XIV

Dialysis of alpha2-M and albumin zinc

Days	Alpha2-M bound Zn µg Zn/mg	Albumin bound Zn µg Zn/mg
1	18.2	9.5
2	14.9	4.5
3	12.0	3.8
4	7.5	2.7
5	5.4	1.5
6	4.8	1.2
7	4.5	1.0

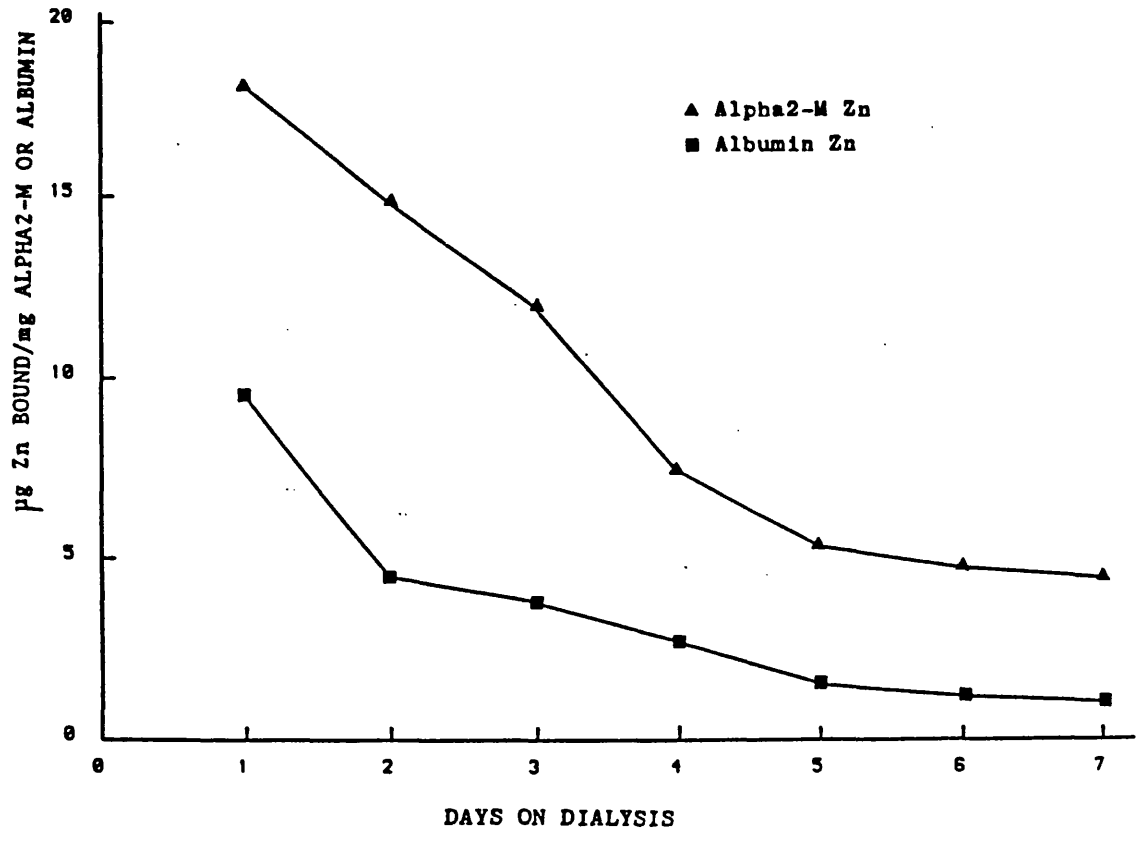


Fig.35 Comparison of Zn-binding to alpha2-M and albumin (B.S.A.) during dialysis for 7 days.

DISCUSSION

Mean plama Zn and Cu concentrations in Cu-deficient sheep (Table-IX) were reduced significantly ($P<0.001$) as observed by Al-Mukhtar (1984)in Cu-deficient sheep. Reduction in plasma Cu was a direct response of dietary Cu-deficiency and absence of any homeostatic mechanism for Cu. Whereas, reduction of Zn in plasma could be due to impairment of absorption at intestinal level. Alfaro and Heaton (1974) suggested that a minimum level of Cu is necessary in the body to facilitate the absorption of Zn. They observed reduced liver Zn concentration in Cu-deficient rats.

In this study, Zn-binding to alpha2-M was studied at two levels of Zn:level-I (Table-X), 150 μg Zn;level-II (Table-XI), 1500 μg Zn. In Cu-deficient sheep alpha2-M incubated with both levels of Zn, showed significantly ($P<0.001$) increased *in vitro* Zn-binding over the first 4 weeks. At Zn level-II (1500 μg Zn), the increased *in vitro* Zn-binding was maintained over the entire 6 weeks, whilst at Zn level-I (150 μg Zn) this increased Zn-binding to alpha2-M was subsequently lost after an initial period of 4 weeks. The reason for this is not clear but it may represent the existence of different binding sites on alpha2-M.

It may also indicate the possibility of altered Zn status

of alpha2-M. This is well demonstrated by the results of Table-XII. Ratio of Zn, Pool-I:Pool-II indicates a shift in Zn from albumin to alpha2-M as the duration of Cu-deficiency was increased.This shift was not so pronounced upto week 3, however,from week 4 pool I Zn was greater than Pool-II even after feeding Cu-supplemented diet.These results suggest that in Cu-deficiency plasma Zn is exchanged from albumin to alpha2-M. As a result of this proposed exchange of Zn between alpha2-M and albumin,*in vitro* Zn-binding was effected at both levels of Zn.

Zn-binding to alpha2-M from sheep fed a Cu-deficient diet may also have been affected by the binding affinities of Zn-binding sites on alpha2-M compared to albumin. Thus when albumin and alpha2-M to which excess Zn (2000 μg Zn) had been added were dialysed against 1 mM tris,pH 7.4 (Table-XIV), alpha2-M showed 50% higher bound Zn than that of albumin after 24 hours and 350% higher than albumin after 7 days of dialysis (Table-XIV, Fig.35). In terms of g-atoms Zn/mol of protein 50 times more Zn was bound to alpha2-M than albumin after 7 days. These results are in general agreement with those of Adham et al (1977) although the binding of Zn to alpha2-M was 150% higher in sheep plasma alpha2-M than in human plasma alpha2-M.

It is reasonable to speculate, in the absence of any

comparable work, that the metabolic stress created by dietary Cu-deficiency might contribute to an increased binding of Zn to alpha2-M. Results in this study, demonstrated significantly ($P<0.001$) increased Zn-binding for 4 weeks at level-I Zn, 6 weeks at level-II Zn and the Zn-binding to alpha2-M increased after Cu supplementation in the diet.

EXPERIMENT-6

EFFECT OF DIETARY HIGH COPPER ON ZINC-BINDING TO ALPHA2-MACROGLOBULIN

INTRODUCTION

The occurrence of chronic copper poisoning has been recognized for many years (Mallory, 1925; Beijers, 1932; Gracey and Todd, 1960; Todd and Thompson, 1963) as a major hazard in the intensive rearing of sheep. High dietary Cu concentration may also cause conditioned Zn-deficiency, e.g. Cu-induced Zn-deficiency of swine (Suttle and Mills, 1966). It also causes accumulation of Cu in sheep liver (Todd, 1969). Bremner et al (1976) showed that Zn supplementation offers an effective protection against Cu-toxicosis. Similarly, Parry et al (1984) confirmed these findings and demonstrated that maintaining the dietary Cu to Zn ratio, 1:4 offered protection against Cu poisoning to three breeds of pregnant ewes maintained indoors.

In Experiment 5 dietary Cu-deficiency increased Zn-binding to alpha2-M from sheep plasma. In order to provide further evidence of the interference of dietary Cu level with Zn-binding to alpha2-M and its dependence on dietary concentration of Cu and Zn; high-Cu diet was

fed to sheep. Zn-binding to alpha2-M in the plasma of sheep fed on high-Cu diet have not been reported so far.

Details of high-Cu diet, alpha2-M purification by IAC and Zn-binding to alpha2-M are described in Section-II.

RESULTS

Mean plasma Zn concentration in high-Cu sheep (Table-XV, Fig.36) decreased weekly starting from week 0. This low concentration of zinc was maintained even after giving Cu-supplemented diet (5mg Cu/kg diet and 50 mg Zn/kg diet) after week 7.

Plasma Zn concentration in this experiment (Table-XV) decreased from a mean of 2.10 μg Zn/ml (at the begining of experiment) to 1.38 μg/ml after week 7. Lowest mean Zn concentration, 0.96 μg Zn/ml was observed after week 4. Bremner et al (1976) observed reduced Zn concentration in sheep fed on a basal diet containing 29 mg Cu/kg diet. This diet was Cu-toxic, and the reduction in mean plasma Zn concentration was much less (9%) observed after 7 weeks. In this study after one week, 22.8% reduction in plasma Zn was observed in high-Cu sheep (Table-XV). After 7 weeks plasma Zn was 34.3% lower than week 0.

Mean plasma Cu concentration in high-Cu sheep increased from 0.73 μg Cu/ml to 3.18 μg Cu/ml (Table-XV, Fig.36) in one week on experimental diet. Cu concentration reduced

Table- XV

Mean Zn and Cu concentrations in high-Cu sheep plasma.

		µg Zn/ ml plasma		µg Cu/ ml plasma	
Week	No.	Mean	S.E.M.	Mean	S.E.M.
0	5	2.10***	± 0.18	0.73***	± 0.04
1	5	1.62***	± 0.04	3.18***	± 0.29
2	5	1.47***	± 0.02	1.65***	± 0.13
3	5	1.15***	± 0.04	1.04***	± 0.09
4	5	0.96***	± 0.03	0.99***	± 0.13
5	5	1.40***	± 0.09	0.93***	± 0.08
6	5	1.35***	± 0.11	0.12 NS	± 0.05
7	5	1.38***	± 0.12	0.08 *	± 0.02
8	5	1.32***	± 0.06	0	
9	5	1.35 **	± 0.23	0	

NS Not significant

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

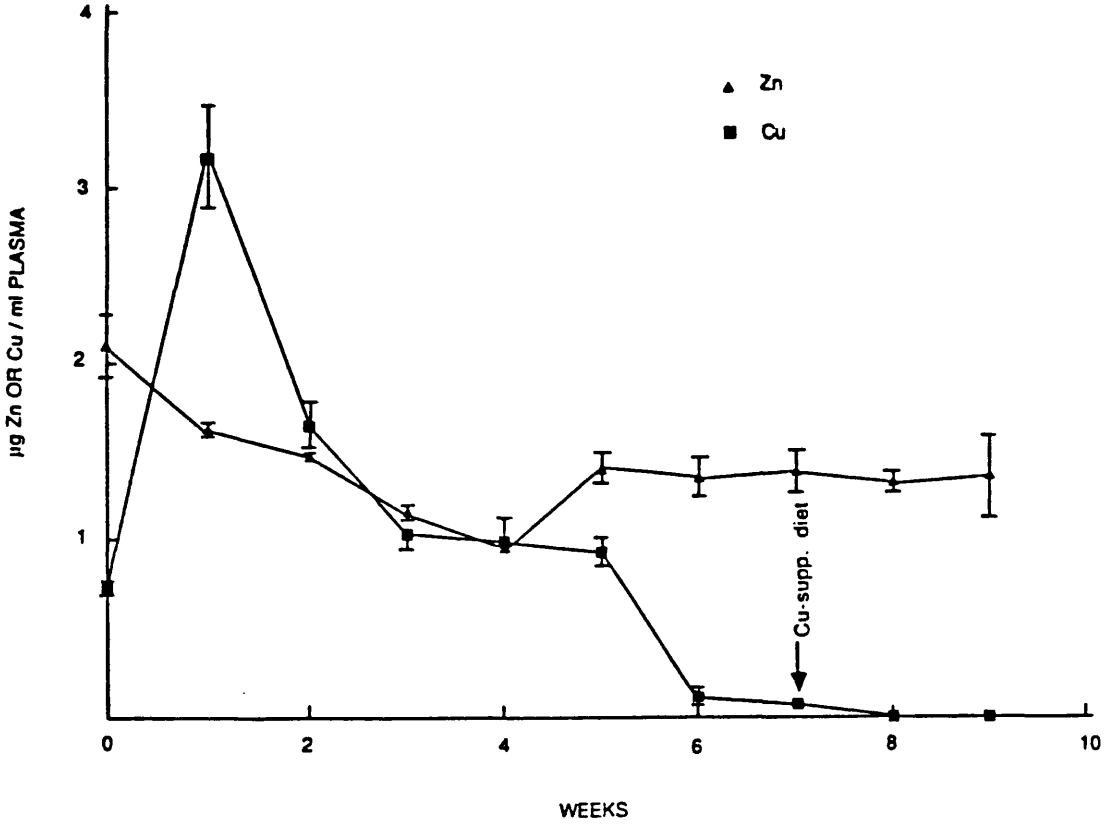


Fig.36 Mean Zn and Cu concentrations in high-Cu sheep plasma.

in week 2 and continued to decrease significantly ($P<0.001$) until week 5. After 6 weeks on high-Cu diet, 2 sheep showed no detectable Cu in their plasma and the other three contained only 0.2 μg Cu/ml plasma (Table-XV). Despite giving normal diet there was no Cu in plasma after weeks 8 and 9. After 9 weeks two sheep died within 24 hours after obtaining blood samples.

Mean plasma Cu concentration increased in one week by 335.6% and after 7 weeks on high-Cu diet decreased by 89% (Table-XV). These results differed from Bremner et al (1976) who showed a gradual increase in Cu concentration from 1.0 μg Cu/ml to about 1.7 μg Cu/ml plasma in 16 weeks. However, they did observe a transient increase in plasma Cu over 2.0 μg/ml.

Results presented in Table-XVI, Fig.37 showed at level Zn (150 μg) incubation, a 86.1% reduction <u>in vitro</u> Zn-binding to alpha2-M. One week after feeding high-Cu diet the Zn bound/100 μg alpha2-M was reduced from 43.10 to 5.98 μg Zn, which was equivalent to $47.80 .10^2$ and $6.63 .10^2$ g-atoms Zn/mol alpha2-M respectively.

During 7 weeks on high-Cu diet (Table-XVI), sheep plasma alpha2-M exhibited a significant ($P<0.001$) reduction <u>in vitro</u> μg Zn bound/100 μg alpha2-M. There was an improvement in Zn-binding after giving normal diet for a further period of 2 weeks to 72.66 μg Zn/100 μg alpha2-M,

Table- XVI

Zn-binding to alpha2-M obtained from high-Cu sheep plasma incubated with 150 µg Zn (Level-I).

Week	No. of assays	µg Zn bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/ mol alpha2-M
0	5	43.10 **	± 7.08	$47.80.10^2$
1	5	05.98 ***	± 0.52	$6.63.10^2$
2	5	10.55 ***	± 1.44	$11.70.10^2$
3	5	19.30 ***	± 1.66	$21.41.10^2$
4	5	27.18 ***	± 0.79	$30.15.10^2$
5	5	39.70 ***	± 1.46	$44.03.10^2$
6	5	42.04 ***	± 1.70	$46.63.10^2$
7	5	21.75 ***	± 1.66	$24.12.10^2$
8	5	29.73 ***	± 1.81	$32.97.10^2$
9	5	72.66 ***	± 2.28	$80.59.10^2$

** $P < 0.01$ *** $P < 0.001$

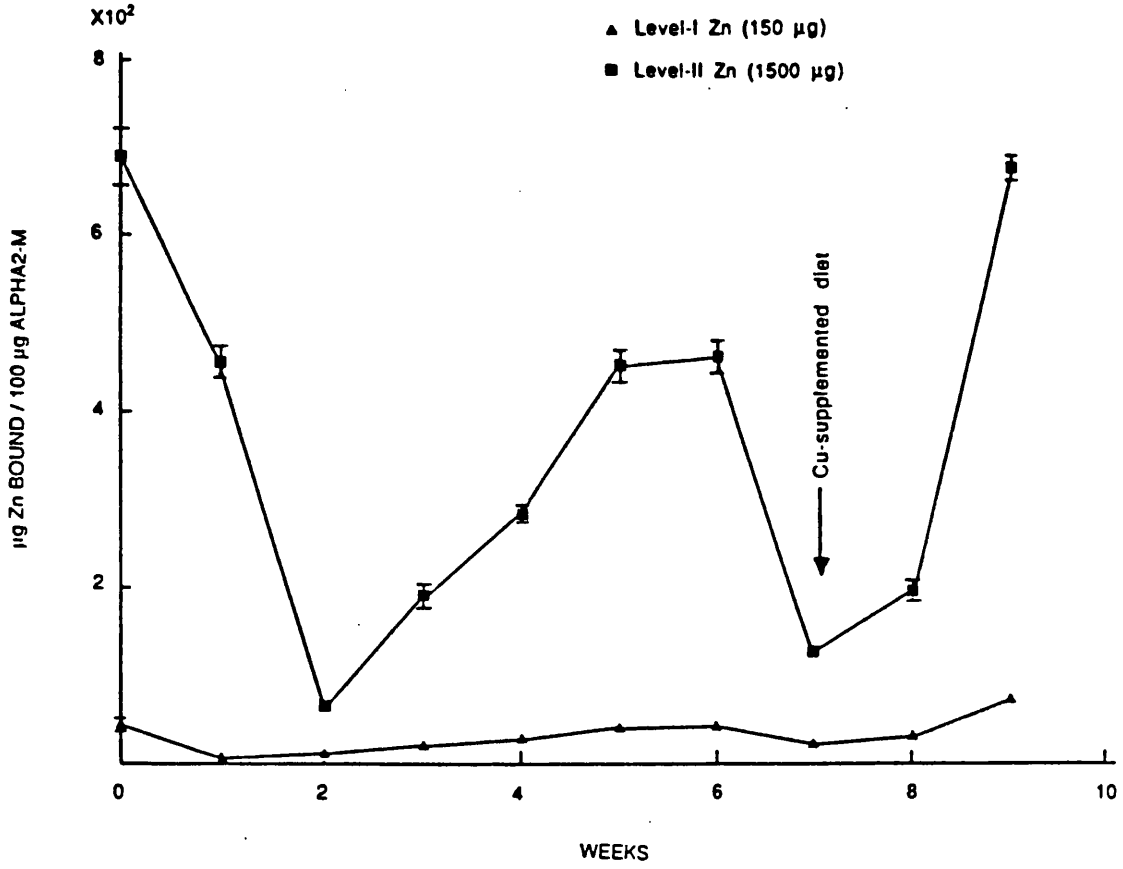


Fig.37 Zn-binding to alpha2-M obtained from high-Cu sheep plasma incubated with two levels of Zn (150 and 1500 µg).

Table- XVII

Zn- binding to alpha2-M obtained from high-Cu sheep plasma incubated with 1500 µg Zn (Level- II).

Week	No. of assays	µg Zn bound/ 100 µg alpha2-M		g-atoms Zn/ mol alpha2-M
		Mean	S.E.M.	
0	5	688.7***	± 32.5	$76.39.10^3$
1	5	455.2***	± 17.7	$50.49.10^3$
2	5	63.5***	± 4.2	$7.04.10^3$
3	5	190.5***	± 13.4	$21.13.10^3$
4	5	284.7***	± 9.4	$31.58.10^3$
5	5	451.5***	± 17.9	$50.08.10^3$
6	5	461.4***	± 18.0	$51.18.10^3$
7	5	127.1***	± 6.7	$14.10.10^3$
8	5	196.8***	± 12.0	$21.83.10^3$
9	5	675.6 ***	± 14.0	$74.93.10^3$

*** $P < 0.001$

which was equivalent to $80.59.10^2$ (Table-XVI, Fig.37).

At the end of 7 weeks on high-Cu diet (Table-XVI), there was a 49.5% reduction *in vitro* of Zn-binding to alpha2-M compared to week 0.

Sheep plasma incubated with level II Zn (1500 µg) showed a 90.8% reduction in Zn-binding to alpha2-M after week 2, from 688.7 to 63.5 µg Zn bound/100 µg alpha2-M and in terms of g-atoms Zn/mol alpha2-M from $76.39.10^3$ to $7.04.10^3$ (Table-XVII and Fig.37). Alpha2-M maintained a significantly ($P<0.001$) reduced *in vitro* Zn-binding throughout 7 weeks on high-Cu diet and at the end of 7 weeks showed a reduction of 81.5% in Zn-binding to alpha2-M compared to that at the begining. Feeding normal diet for a period of further 2 weeks increased Zn-binding and attained nearly the same level as at the begining of the experiment, 688.7 and 675.6 µg Zn/100 µg alpha2-M respectively.

Plasma from sheep fed on high-Cu diet showed (Table-XVIII, Fig.38) decreased Zn concentration in alpha2-M pool (Pool-I) Zn and albumin pool (Pool-II) Zn throughout the experimental period including substituting normal diet during the weeks 8 and 9. Only exception to this was an increase of alpha2-M pool Zn after 6 weeks. Zn bound to Pool-II was maintained higher than Pool-I during the 7 weeks on high-Cu diet.

Table- XVIII

Zn concentration of alpha2-M pool (Pool-I) and albumin pool (Pool-II) in high-Cu sheep plasma.

µg Zn/ ml plasma

Week	Pool-I	Pool-II	Ratio of Zn I:II
0	0.570	1.550	0.36:1
1	0.512	1.182	0.43:1
2	0.470	1.040	0.45:1
3	0.310	0.835	0.37:1
4	0.320	0.650	0.49:1
5	0.240	0.932	0.26:1
6	0.580	0.908	0.63:1
7	0.530	0.915	0.58:1
8	0.450	0.950	0.47:1
9	0.500	0.870	0.57:1

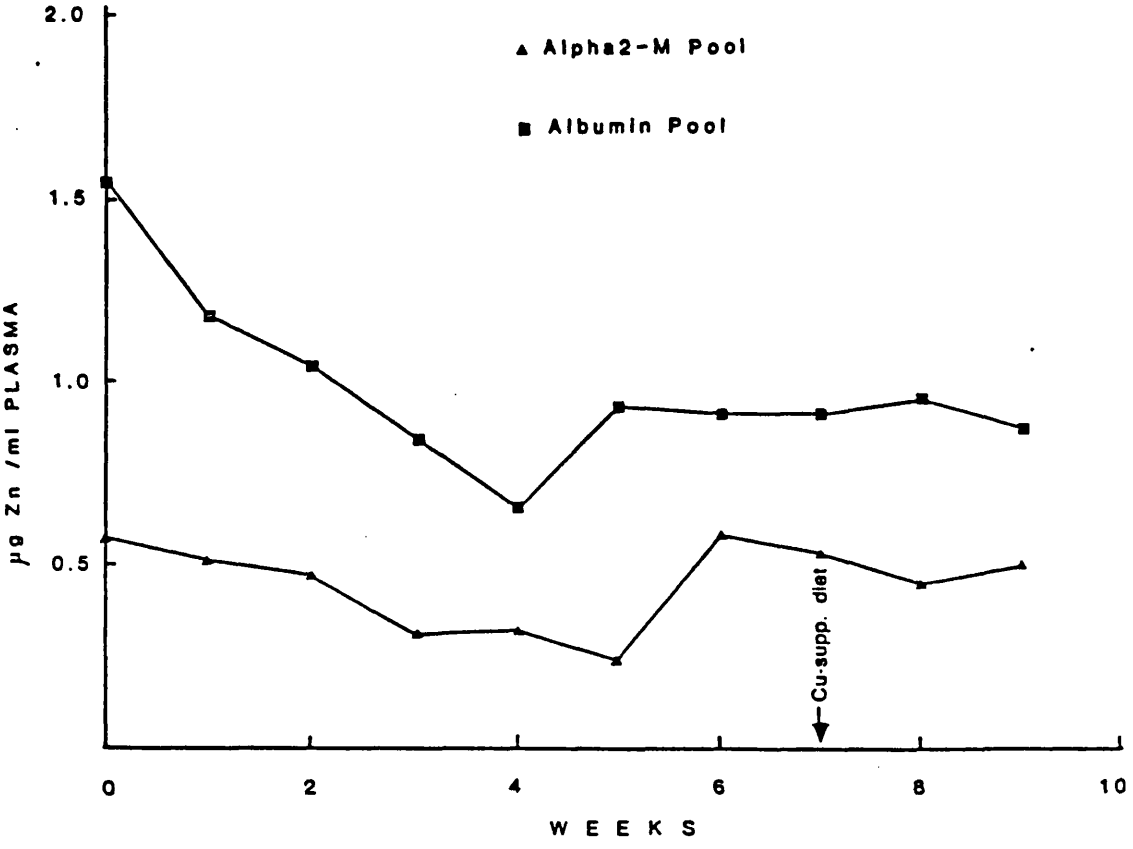


Fig.38 Zn concentration of alpha2-M pool (Pool-I) and albumin pool (Pool-II) in high-Cu sheep plasma.

At the end of 7 weeks alpha2-M pool Zn was only 7% less than at the begining of week 0. However, albumin pool Zn reduced by 41% from week 0 (Table-XVIII).

At the start of the experiment purified alpha2-M Zn concentration was 0.440 mg/g alpha2-M and there was no detectable Cu present (Table-XIX, Fig.39). After one week on high-Cu diet, not only Cu was bound to alpha2-M but the Zn concentration increased nearly 2.4 times. During the period of 7 weeks on high-Cu diet, alpha2-M Cu varied between 0-0.400 mg/g alpha2-M and at the end of 7 weeks there was no Cu present in alpha2-M (Table-XIX, Fig.39).

Table- XIX

Zn and Cu concentrations in alpha2-M obtained from high-Cu sheep plasma.

Week	mg Zn/g alpha2-M	mg Cu/g alpha2-M
0	0.440	-
1	1.040	0.200
2	1.160	0.160
3	1.140	0.040
4	1.330	0.080
5	1.300	0.240
6	1.300	0.400
7	0.650	-
8	0.500	-

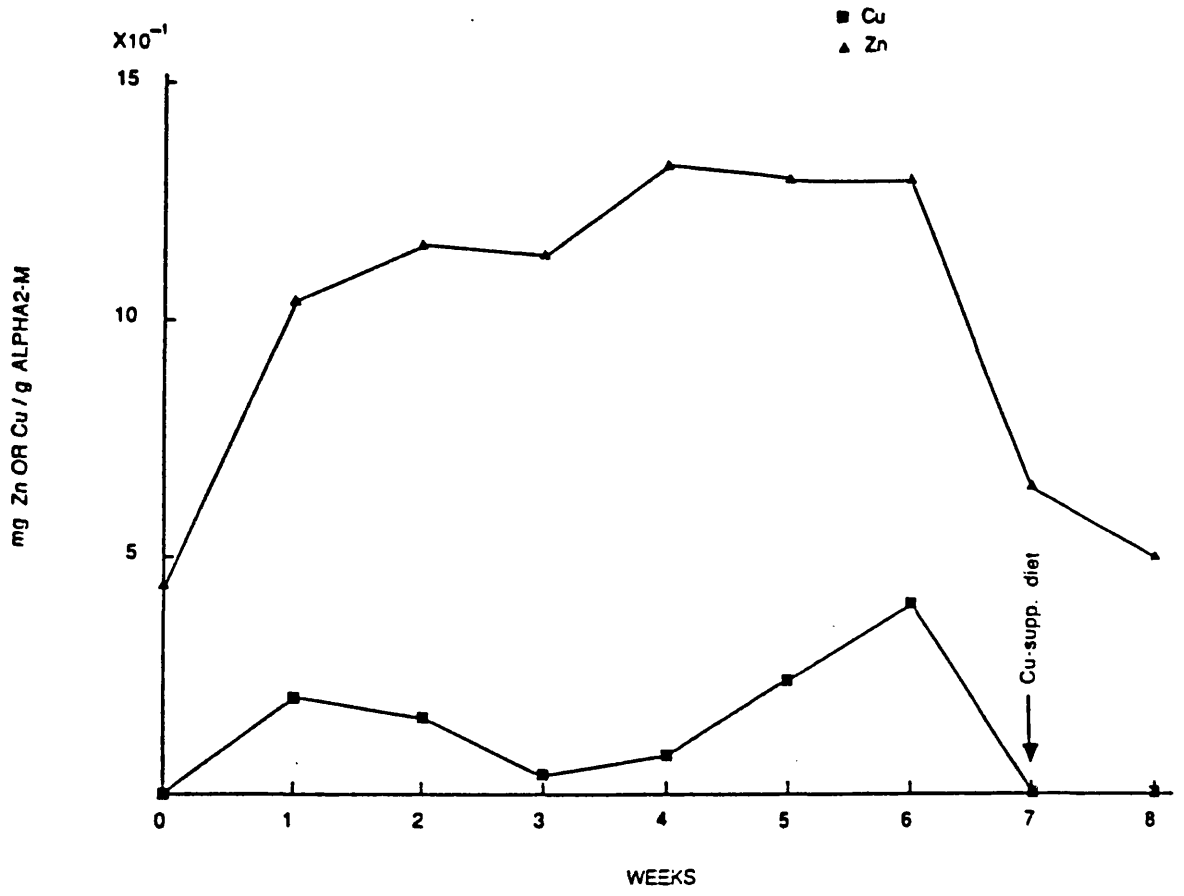


Fig.39 Zn and Cu concentrations of alpha2-M obtained from high-Cu sheep plasma.

DISCUSSION

The most interesting results from this experiment are shown in Table-XIX. Firstly, alpha2-M in sheep fed a high-Cu diet, was binding Cu after one week on the feeding regime. Secondly, the amount of alpha2-M bound Cu increased from 0.2 mg to 0.4 mg Cu/g alpha2-M over a period of six weeks. Thirdly, whilst alpha2-M was showing increased quantity of bound Cu, the amount of bound Zn also increased over the six week period from 0.44 mg at week 0, to 1.3 mg Zn/g alpha2-M.

These results showed that alpha2-M could bind Cu in the presence of Zn; furthermore, as the ingested Zn was increased, a substantial quantity of Zn was correspondingly bound to alpha2-M as well as substantially increased bound Cu. Therefore, alpha2-M was shown to have a much greater capacity for binding Zn than has previouly been recorded as compared by control values at week 0, and this increased Zn-binding was occuring simultaneously with substantially increased Cu-binding; this was a surprising result, especially in the light of the fact that alpha2-M does not normally bind Cu as shown in week 0.

It is difficult to speculate on the molecular mechanism of accommodating the increased Cu-binding simultaneously with increased Zn. If the amount of bound Zn in alpha2-M

had shown a quantitative decrease in the presence of the increased bound Cu, then it would have been reasonable to reflect on Zn-binding sites accommodating the Cu. However, until further work can be carried out to test the strength of binding and specificity of such binding sites for either Zn or Cu, it can only be suggested at this stage that more binding sites for Zn and Cu are available on alpha2-M than has previously been envisaged.

In contrast to results shown in Table-XVIII, which showed evidence of antagonism between dietary Cu and Zn in alpha2-M and albumin fractions, the results in Table-XIX do not immediately confirm this antagonism. It was expected that if such antagonism existed that the alpha2-M Zn in Table-XIX would have decreased at the expense of the increased alpha2-M Cu levels. However, it is reasonable that antagonism was present in the results of Table-XIX, but due to the substantial and abnormal quantities of ingested Zn, that such an antagonism between Cu and Zn in alpha2-M, was masked.

Significantly ($P<0.001$) reduced plasma Zn concentratios (Table-XV) were observed in high-Cu sheep plasma and the decreases were gradual. The greater Zn reduction (34.3%) after 7 weeks compared to 9% reduction of Zn after 9 weeks in the studies of Bremner et al (1976) may be due to the differences in dietary Cu concentration of 250 mg

Cu/kg diet in this study and 29 mg Cu/kg diet in their work. It may also reflect differences in the Cu to Zn ratio. In this study it was was 5:4 and in their experiments 1:1.5 (Cu:Zn). This may explain the fact that the inclusion of Zn at 200 mg/kg diet in this study was unable to protect against the effect of Cu-toxicosis. Bremner et al (1976) also used higher ratios of Cu:Zn, 1:7.6 and 1:14.5 which offered greater and effective protection against Cu-toxicosis.

The Cu level rising fast during the first week from 0.73 to 3.18 μg Cu/ml plasma (a 335.6% increase). This effect was a consistent finding and has been shown in previous experiments from this laboratory (Al-Mukhtar, 1984). From week 2 the Cu concentration decreased gradually showing 89% decrease after 7 weeks. There was no Cu present in plasma in weeks 8 and 9. The results in Table-XV differed from Bremner et al (1976). The ingested Cu was probably accumulated in liver as shown by Todd et al (1962 and 1963), Ishmael et al (1971 and 1972). Liver Cu was not assayed in this study, but, when two sheep died after completion of the experiment, post-mortem confirmed the cause of death as copper poisoning and accumulation of Cu in the liver. Results in this study are in agreement with Todd et al (1962 and 1963), Ishmael et al (1971 and 1972) on the accumulation of Cu in liver in Cu-toxicosis. However, these studies did not report the concetration of Zn in their diets, which makes it difficult to compare

the results. Dietary Zn concentration plays a vital role and the effects of dietary high-Cu depend upon the level of Zn in the diet as shown by Bremner et al (1976) and Parry et al (1984). This is supported by the differences observed in this study.

Zn-binding to alpha2-M in high-Cu sheep plasma decreased on incubation with both levels of Zn (150 and 1500 μg Zn) (Table-XVI & XVII). The decrease was rapid with level I Zn than with level II. This decrease in Zn-binding to alpha2-M may be due to the combined effect of dietary Cu-excess and greater affinity of Cu to binding sites on alpha2-M. Recent evidence presented by Gettins and Cunningham (1986) showed by NMR study, that manganese binds to human apo-alpha2-M analogously to Zn and this evidence showed the possibility of Cu replacing Zn on some weaker sites.

The results in this study indicated three main features of <u>in vitro</u> Zn-binding to alpha2-M in high-Cu sheep plasma. First, Zn-binding to alpha2-M decreased, this was followed by an increase, but after 7 weeks Zn-binding was significantly ($P<0.001$) decreased. Lastly, feeding a normal diet has shown increased Zn-binding to alpha2-M.

Cu has greater affinity than Zn for the available binding sites on proteins (Bremner et al, 1974; Williams, 1984). The combined effect of high dietary Cu and the greater

affinity may be responsible for lowered Zn-binding to alpha2-M. Bremner et al (1974) stressed the importance of understanding the relatioship between Cu and Zn and their distribution among liver proteins to explain the mutual antagonism between Cu and Zn in animals. Similarly, Williams (1984) suggested that transfer of Cu cannot be entirely dependent on binding strengths and thermodynamics alone, since that of Cu is always greater than that of Zn. Suttle and Mills (1966) showed that high dietary Cu induced Zn-deficiency in swine. The combined effect of the above considerations and the evidence in support of these was provided by the results in this study and it can be explained as follows (Fig.40):

The influence of high-Cu diet was to increase the plasma Cu concentration and decrease both the plasma Zn concentration and the <u>in vitro</u> Zn-binding to alpha2-M. Although liver Cu was not measured in this study, there was accumulation of Cu in liver (as shown by postmortem indicating Cu poisoning and accumulation of Cu in liver). Accumulation of Cu in liver was due to transfer of Cu from plasma to liver. Occurrence of this event was supported by decreased Cu concentrations in plasma and increased <u>in vitro</u> Zn-binding to alpha2-M. However, this transfer of Cu to liver may be dependent on the rate of Cu accumulation in liver, which was supported by the evidence of varied Zn-binding to alpha2-M. On feeding a normal diet to sheep after 7 weeks Zn-binding to alpha2-M

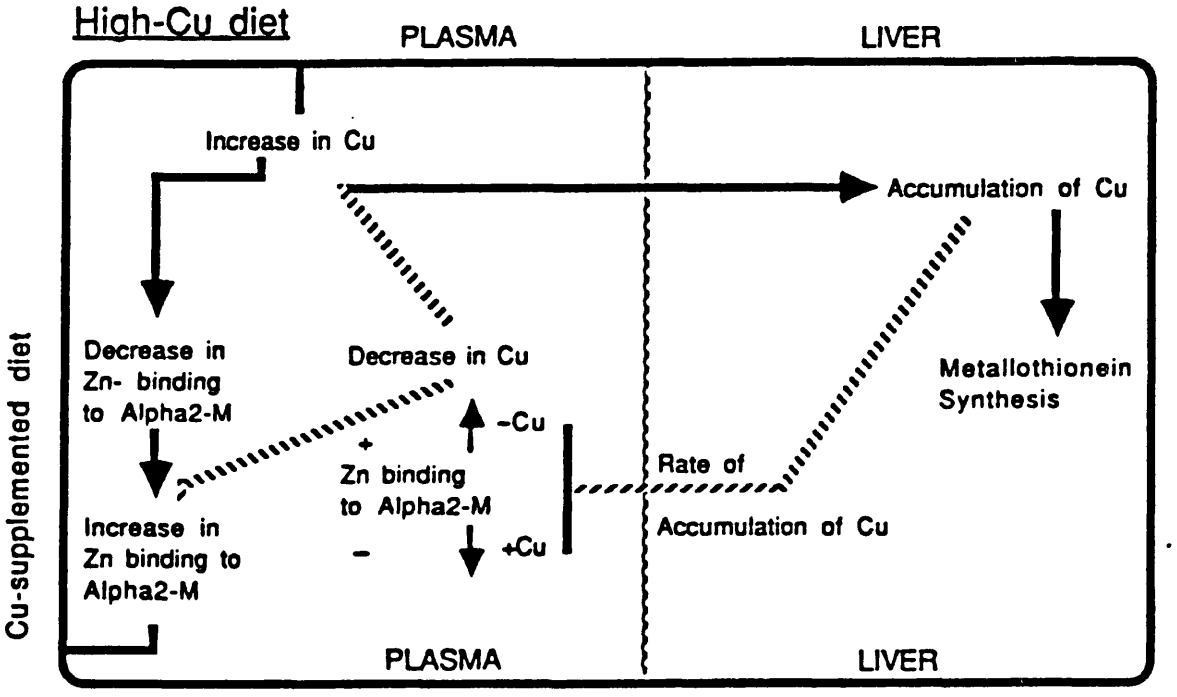


Fig.40 Distribution of Cu - Metallothionein Sythesis - Zn - binding to Alpha2-M

increased indicating lack of transfer of Cu to liver.

The results in Table-XVIII showed decreased Zn concentration in alpha2-M pool (Pool-I) and albumin pool (Pool-II). In comparison with Pool-II Zn decreae in Pool-I Zn after 7 weeks by only 7% to that on Week 0 indictes the greater binding affinity for Zn by alpha2-M. Whereas, 41% reduction in Pool-II Zn after 7 weeks compared to Week 0 indicates weaker binding affinity than alpha2-M. However in both pools Zn was lowered as a direct response to high-Cu diet and probably diminished absorption of Zn at the intestine.

It is suggested (Evans, 1973) that chemical similarity of Cu and Zn leads to competition between these elements at various binding sites within specific metabolic systems, including intestinal absorption. Van Campen and Scaife (1967) demonstrated that Zn interacts with Cu at a site either in or on the intestinal mucosa. These studies support the possibility of interference of Zn-absorption by high dietary Cu concentration.

Recovery of Zn in both the pools may be dependent on the rate of accumulation of Cu in liver and limiting factors in the accumulation of Cu and the synthesis of metallothionein in liver as shown in Fig.40. In vitro Zn-binding to alpha2-M reflected any changes in Zn status of alpha2-M as shown in Fig.40.

EXPERIMENT-7

EFFECT OF VARIABLE Cu AND Zn CONCENTRATIONS ON Zn-BINDING TO ALPHA2-MACROGLOBULIN IN THREE BREEDS OF PREGNANT EWES

INTRODUCTION

Copper toxicity has long been recognised as a major hazard in the intensive rearing of sheep (Bremner et al,1976). This syndrome is always associated with a high concentration of liver Cu concentration but no definite correlation has been achieved between Cu concentration in the diet and liver Cu. Van der Schee et al (1983) linked earlier evidence of Bremner et al (1976) of Zn as dietary antagonist to Cu,with the possibility that the effect of a ratio of these metals may differ between breeds.

In Experiments 5 and 6, *in vivo* Cu level has interfered with *in vitro* Zn-binding to alpha2-M and it was dependent on the level of Zn and Cu in the diet. In order to provide further evidence of the interference of Cu with Zn-binding to alpha2-M and its dependence on dietary concentration of Zn and Cu, samples of blood were obtained from three breeds of pregnant ewes fed on diet containing Cu,Zn,1:4 and kept indoors. This experiment was carried out to investigate the breed differences *in vitro* Zn-binding to alpha2-M.

RESULTS

It is interesting to note from Table-XX that in Dorset ewes the mean plasma Cu concentration on Day-0, which was the first day of housing, was higher than at any subsequent sampling day. The mean plasma Cu value before housing took place for the Dorset group was 1.84 μg Cu/ml plasma significantly lower ($P<0.001$) than Day-0. This showed that the effect of housing on pregnant Dorset ewes increased the plasma Cu concentration.

The subsequent mean plasma Cu concentration in Dorset ewes decreased at first and was variable upto 86 days.

The mean plasma Zn concentration in Table-XX before housing was 1.26 μg/ml. On day-0 of housing showed a significant ($P<0.001$) decrease to 0.99 μg/ml plasma, and continued to decrease in pregnant Dorset ewes from 0.99 μg/ml plasma on day-0 to 0.70 μg/ml plasma, significantly ($P<0.001$) upto 55 days. After which increased gradually upto 86 days, when the mean Zn was 1.13 μg/ml plasma (Table-XX, Fig.41).

The mean plasma Cu concentration (1.43 μg/ml plasma) in pregnant Clun ewes in Table-XX1, Fig.42 showed significant ($P<0.01$) increase on Day-0 compared to Cu concentration (1.18 μg Cu/ml) prior to housing. The effect of housing pregnant Clun ewes increased the plasma Cu. At first Cu concentration indoors decreased

Table- XX

Mean Cu and Zn concentrations in the plasma of pregnant Dorset ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4.

		µg Cu/ml plasma		µg Zn/ml plasma	
Days	No.	Mean	S.E.M.	Mean	S.E.M.
0	10	2.31***	± 0.13	0.99***	± 0.07
35	10	1.55***	± 0.09	0.82***	± 0.05
42	10	1.22***	± 0.07	0.75***	± 0.03
55	10	1.15***	± 0.06	0.70***	± 0.03
69	10	1.31***	± 0.06	0.78***	± 0.03
74	10	1.20***	± 0.06	0.90***	± 0.02
86	10	1.56***	± 0.06	1.13***	± 0.02

***$P < 0.001$

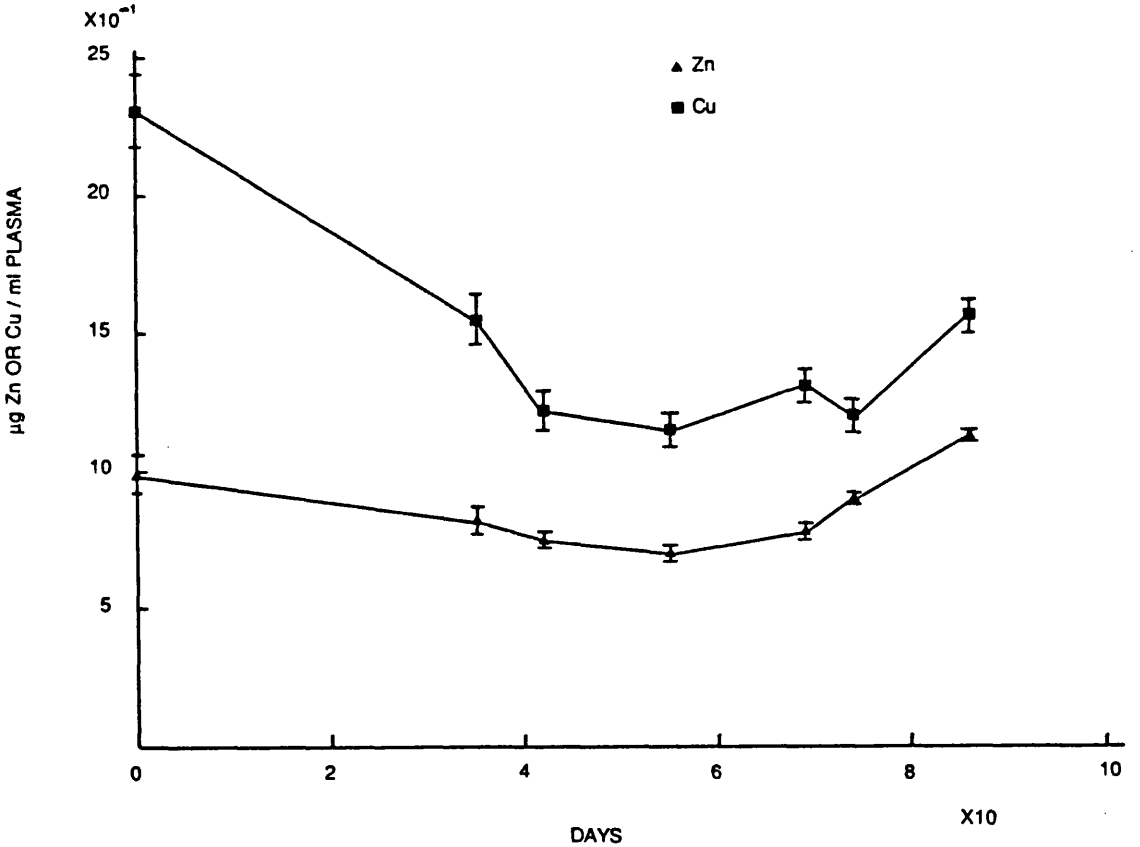


Fig.41 Mean Zn and Cu concentrations in the plasma of pregnant Dorset ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4.

Table- XXI

Mean Cu and Zn concentrations in the plasma of pregnant Clun ewes maintained indoors on vairiable Cu and Zn diet in the ratio 1:4.

		µg Cu/ml plasma		µg Zn/ml plasma	
Days	No	Mean	S.E.M.	Mean	S.E.M.
0	10	1.43***	± 0.08	0.66***	± 0.01
14	10	1.17***	± 0.06	0.86***	± 0.03
21	10	1.21***	± 0.05	0.69***	± 0.03
35	10	1.29***	± 0.03	0.71***	± 0.03
40	10	1.00***	± 0.03	0.86***	± 0.03
48	10	1.32***	± 0.04	0.69***	± 0.03
62	10	1.36***	± 0.02	0.68***	± 0.04

*** $P < 0.001$

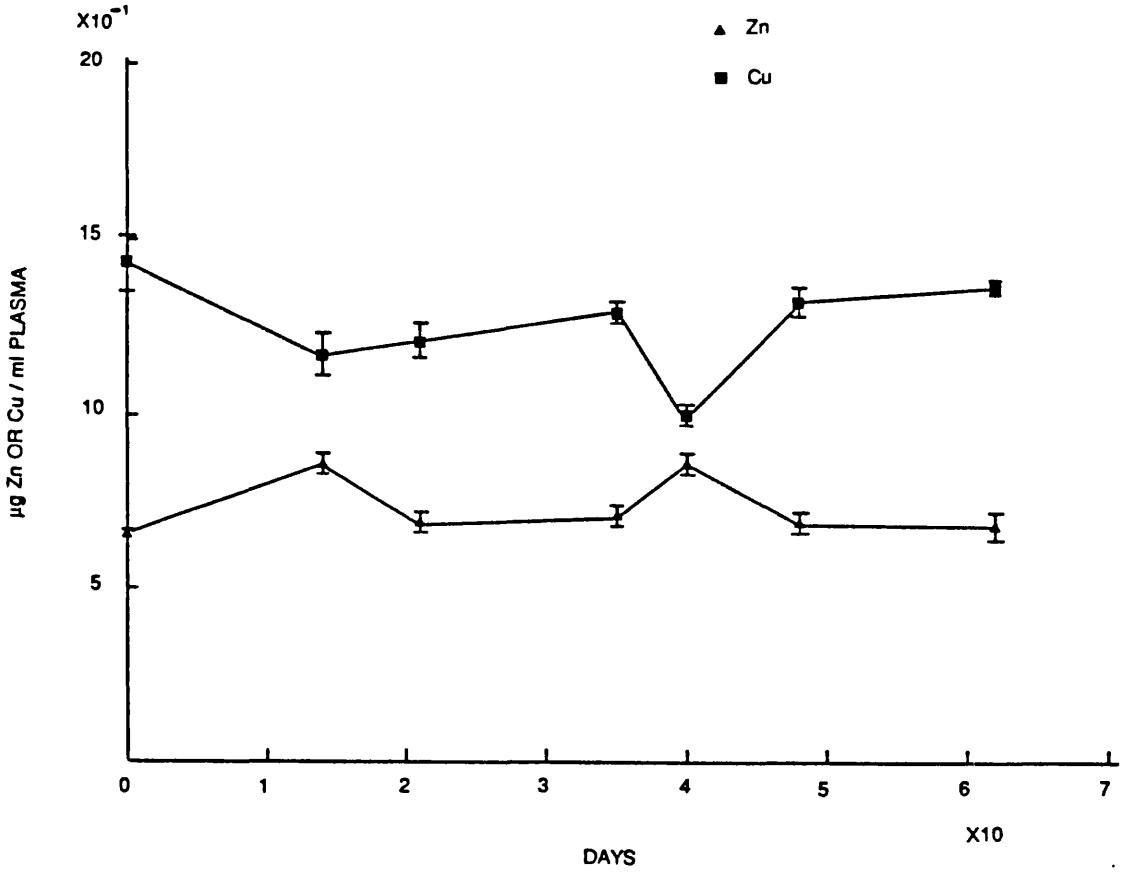


Fig.42 Mean Zn and Cu concentrations in the plasma of pregnant Clun ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4.

significantly (P<0.001), but it was variable during the 62 days (Table-XXI, Fig.42).

The mean Zn concentration in the plasma of pregnant Clun ewes was lowered (Table-XXI) significantly (P<0.001) after housing and remained variable and lower than the concentration observed prior to housing (0.92 μg Zn/ml plasma).

The mean plasma Cu concentration in the plasma of pregnant Finn ewes (Table-XXII, Fig.43) on day-0 (1.14 μg/ml plasma) was not significantly different to that of the concentration (1.16 μg Zn/ml plasma) prior to housing. However, a significant (P<0.001) reduction in the plasma concentration was observed following housing and feeding on the variable Cu and Zn diet. Subsequently, the Cu concentration increased during 62 days.

The mean Zn concentration in the plasma of pregnant ewes decreased significantly (P<0.001) after housing compared to the concentration prior to housing (1.3 μg Zn/ml plasma) and remained variable during 62 days (Table-XXII, Fig.43).

In Table-XXIII, Fig.44 Zn-binding to alpha2-M (incubated with 150 μg Zn) from the plasma of pregnant Dorset ewes increased significantly (P<0.001) from 17.97 to 174.71 μg Zn bound/100 μg alpha2-M or in g-atoms Zn/mol alpha2-M

Table- XXII

Mean Cu and Zn concentrations in the plasma of pregnant Finn ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4.

		µg Cu/ml plasma		µg Zn/ml plasma	
Days	No	Mean	S.E.M.	Mean	S.E.M.
0	10	1.14***	± 0.16	0.98***	± 0.05
14	9	0.91***	± 0.18	0.60***	± 0.04
40	10	0.89***	± 0.09	0.97***	± 0.03
48	10	1.05***	± 0.08	0.77***	± 0.04
62	10	1.18***	± 0.06	0.97***	± 0.04

*** P < 0.001

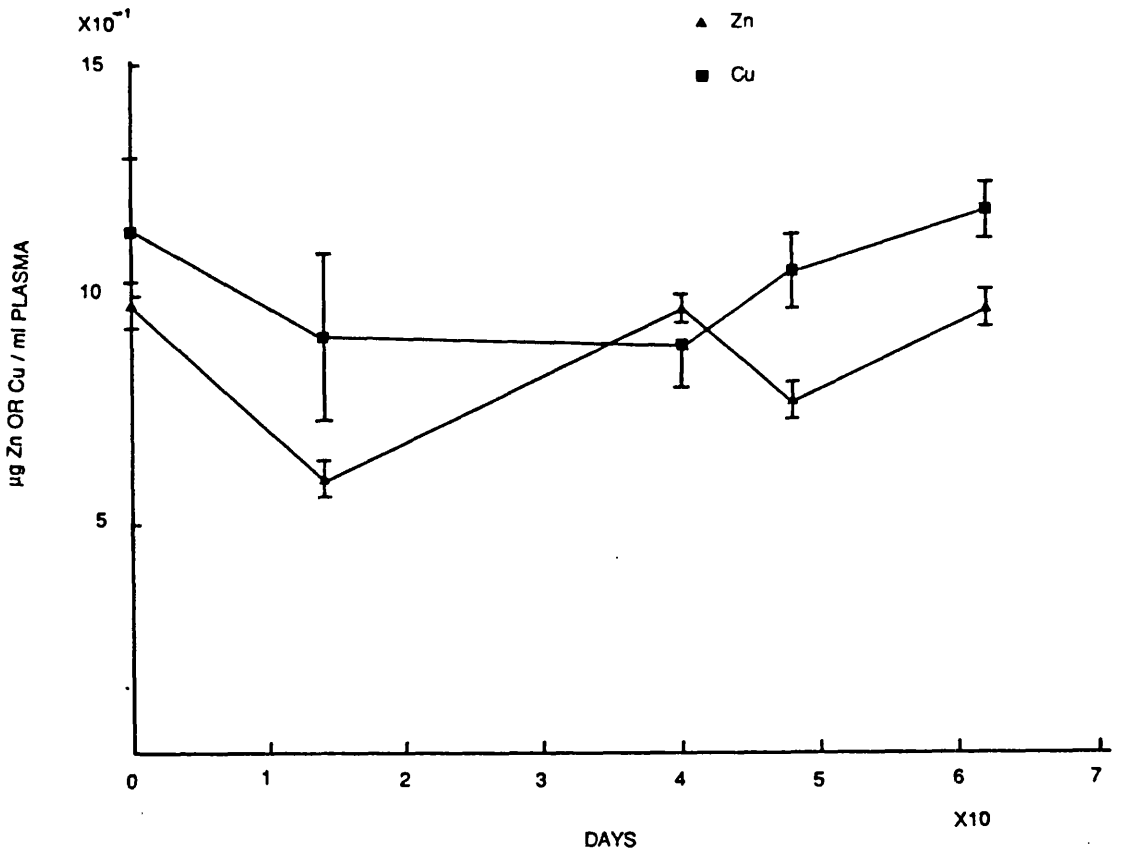


Fig.43 Mean Zn and Cu concentrations in the plasma of pregnant Finn ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4.

Table-XXIII

In vitro Zn-binding to alpha2-M from the plasma of pregnant Dorset ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4.
Aplha2-M incubated with 150µg Zn

Days	No	µg Zn bound/ 100µg Alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	17.97 ***	±3.14	$19.93.10^2$
35	5	174.71 ***	±11.06	$193.78.10^2$
42	5	73.74 **	±11.33	$81.79.10^2$
55	5	44.39 ***	±2.42	$49.23.10^2$
69	5	72.87 ***	±3.68	$80.82.10^2$
74	5	49.98 ***	±5.71	$55.43.10^2$
86	5	16.80 ***	±3.68	$18.63.10^2$

** $P < 0.01$ *** $P < 0.001$

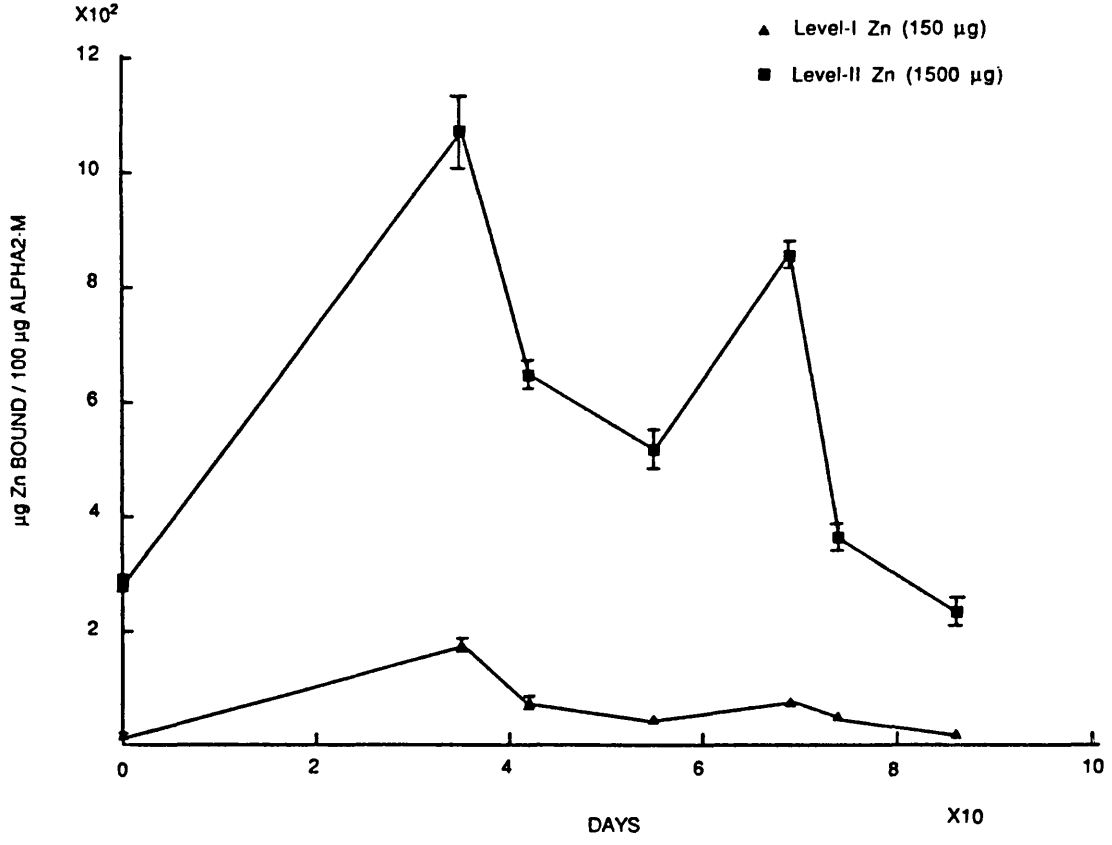


Fig.44 Zn-binding to alpha2-M obtained from the plasma of pregnant Dorset ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4. Alpha2-M incubated with two levels of Zn (150 and 1500 ug).

Table -XXIV

In vitro Zn-binding to alpha2-M from the plasmaof pregnant Dorset ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4.
Alpha2-M incubated with 1500 µg Zn

Days	No	µg Zn Bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	285.60 ***	±13.60	$31.68.10^3$
35	5	1068.30 ***	±63.0	$118.49.10^3$
42	5	646.80 ***	±24.5	$71.74.10^3$
55	5	517.00 ***	±33.90	$57.34.10^3$
69	5	854.20 ***	±23.50	$94.74.10^3$
74	5	363.60 ***	±23.40	$40.33.10^3$
86	5	24.70 ***	±24.70	$25.95.10^3$

*** $P < 0.001$

from 19.93 $.10^2$ to 193.78 $.10^2$ during the first 35 days. However, during 86 days Zn-binding to alpha2-M was variable and after 86 days it was nearly the same as Day-0.

In Table-XXIV, Fig.44 alpha2-M incubated with 1500 µg Zn showed significantly ($P<0.001$) increased Zn-binding, from 285.6 to 1068.3 µg Zn bound/100 µg alpha2-M or in terms of g-atoms Zn/mol alpha2-M increased from 31.68 $.10^3$ to 118.49 $.10^3$ after 35 days on variable Cu and Zn diet. Zn-binding to alpha2-M reduced significantly ($P<0.001$) during 86 days but was variable. However, at the end of 86 days Zn-binding was lowered significantly ($P<0.001$) compared to Day-0.

In Table-XXV, Fig.45 in vitro Zn-binding to alpha2-M incubated with 150 µg Zn decreased significantly ($P<0.001$) in pregnant Clun ewes, from 69.9 to 9.6 µg Zn bound/100 µg alpha2-M or in terms of g-atoms Zn/mol alpha2-M from 77.53 $.10^2$ to 10.65 $.10^2$ during 48 days. After 62 days the Zn-binding was significantly ($P<0.05$) lower than the Day-0.

In Table-XXVI, Fig.45 alpha2-M incubated with 1500 µg Zn showed significantly ($P<0.001$) decreased Zn-binding in pregnant Clun ewes. Zn-binding to alpha2-M was variable during 62 days, however, after 62 days it was 313.8 compared to 492.2 µg Zn bound/100 µg alpha2-M on Day-0.

Table–XXV

In vitro Zn-binding to alpha2-M from the plasma of pregnant Clun ewes maintained indoors on variable Cu and Zn with a ratio 1:4.
Alpha2-M incubated with 150 µg Zn

Days	No	µg Zn bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	69.90 **	±14.10	$77.53.10^2$
14	5	24.60***	±1.10	$27.28.10^2$
21	5	24.40***	±0.70	$27.06.10^2$
35	5	21.00***	±1.60	$23.29.10^2$
40	5	10.00***	±.0.90	$11.09.10^2$
48	5	9.60 ***	±0.70	$10.65.10^2$
62	5	14.10 *	±3.90	$15.64.10^2$

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

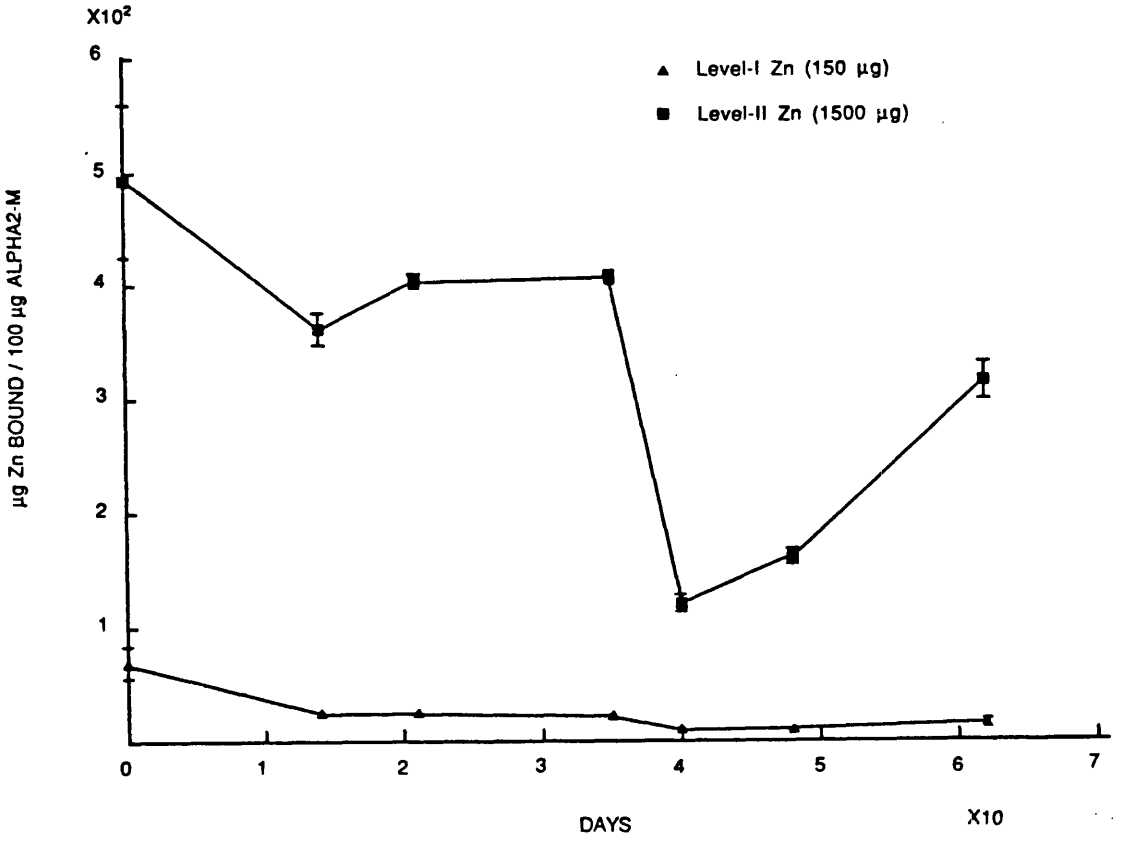


Fig.45 Zn-binding to alpha2-M obtained from the plasma of pregnant Clun ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4. Alpha2-M incubated with two levels of Zn (150 and 1500 µg).

Table-XXVI

In vitro Zn binding to alpha2-M from the plasma of pregnant Clun ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4. Alpha2-M incubated with 1500 µg Zn

Days	No	µg Zn Bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	492.20 ***	±67.20	$54.59.10^3$
14	5	360.50 ***	±14.10	$39.98.10^3$
21	5	402.30 ***	±6.40	$44.62.10^3$
35	5	405.20 ***	±5.60	$44.94.10^3$
40	5	120.50 ***	±7.50	$13.36.10^3$
48	5	160.80 ***	±6.80	$17.83.10^3$
62	5	313.82 ***	±16.50	$34.81.10^3$

*** $P < 0.001$

In terms of the g-atoms Zn/mol of alpha2-M decreased to 34.81 $.10^3$ from 54.59 $.10^3$.

In Table-XXVII, Fig.46 <u>in vitro</u> Zn-binding to alpha2-M incubated with 150 μg Zn in pregnant Finn ewes decreased during 62 days and showed variable binding with corresponding variable significance levels. After 62 days Zn-binding to alpha2-M was significantly ($P<0.001$) lower, 19.93 compared to 31.38 μg Zn bound/100 μg alpha2-M on the Day-0. In terms of g-atoms Zn/mol alpha2-M decreased from 34.80 $.10^2$ to 22.10 $.10^2$.

In Table-XXVIII, Fig.46 <u>in vitro</u> Zn-binding to alpha2-M incubated with 1500 μg Zn, decreased significantly ($P<0.001$) in pregnant Finn ewes, from 349.0 to 93.3 μg Zn bound/100 μg alpha2-M or in terms of g-atoms Zn/mol alpha2-M from 38.71 $.10^3$ to 18.04 $.10^3$ after 14 days on variable Cu and Zn diet. Zn-binding was variable during 62 days and the decrease was significant ($P<0.001$) throughout.

Table-XXVII

In vitro Zn-binding to alpha2-M from plasma of pregnant Finn ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4.
Alpha2-M incubated with 150 µg Zn

Days	No	µg Zn bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	31.38 ***	± 3.99	$34.80.10^2$
14	5	11.56 **	± 1.95	$12.82.10^2$
40	5	20.66 ***	± 1.25	$22.91.10^2$
48	5	7.41 **	± 1.65	$8.22.10^2$
62	5	19.93 ***	± 1.15	$22.10.10^2$

** $P < 0.01$ *** $P < 0.001$

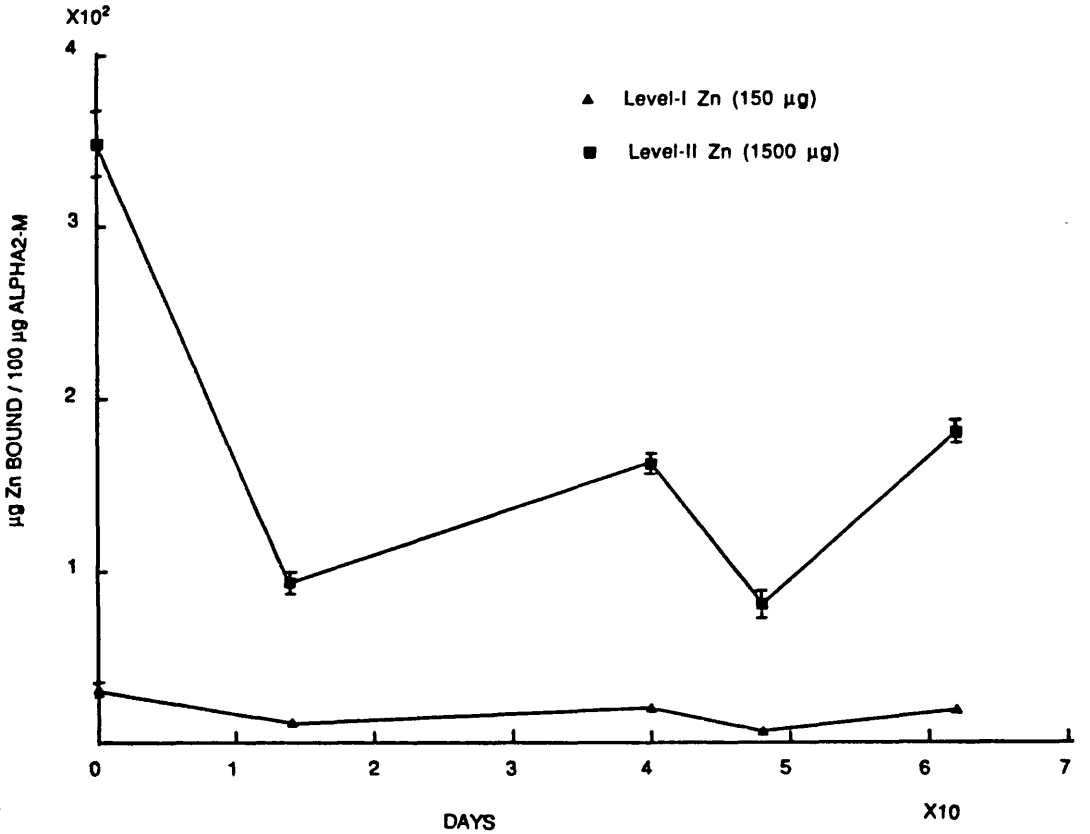


Fig.46 Zn-binding to alpha2-M obtained from the plasma of pregnant Finn ewes maintained indoors on variable Cu and Zn diet in the ratio 1:4. Alpha2-M incubated with two levels of Zn (150 and 1500 µg).

Table-XXVIII

In vitro Zn-binding to alpha2-M from the plasma of pregnant Finn ewes maintained indoors on variable Cu and Zn diet with a ratio 1:4.
Alpha2-M incubated with 1500 µg Zn

Days	No	µg Zn bound/ 100 µg alpha2-M Mean	S.E.M.	g-atoms Zn/mol alpha2-M
0	5	349.00 ***	± 19.20	$38.71.10^{3}$
14	5	93.30 ***	± 6.40	$10.35.10^{3}$
40	5	162.70 ***	±5.7	$18.04.10^{3}$
48	5	81.80 ***	±8.1	$9.07.10^{3}$
62	5	180.60 ***	±6.5	$20.03.10^{3}$

*** $P < 0.001$

DISCUSSION

The effect of housing on pregnant ewes increased the mean plasma Cu concentration in all breeds studied (Tables-XX-XXII), with a significantly ($P<0.001$) greater increase in Dorset ewes (Table-XX) than either Clun or Finn ewes.On the first day of housing mean Zn concentration in plasma of all breeds decreased significantly ($P<0.001$) as shown in Tables-XX-XXII. Decrease in Zn concentration may be directly related to increased Cu in plasma similar to the effect of high-Cu diet as observed by Saylor and Leach (1980) in sheep and Gipp et al (1974) in young pigs. Sheep fed on 47 mg Cu and 46 mg Zn/kg of diet showed decreased Zn concentration in plasma, but they were not significant (Saylor and Leach, 1980). However,in young pigs, dietary Cu of 240 mg/kg showed significantly ($P<0.01$) decreased plasma Zn (Gipp et al, 1974).

Concentrations of both Cu and Zn varied in response to dietary variation of Cu and Zn levels, but maintaining the dietary ratio, 1:4 between these two elements. These variations in plasma Cu and Zn may be related to Cu accumulation in liver and the rate of accumulation may be the controling factor.

Weiner et al (1978) reported the importance of

considering genetic variation in the Cu metabolism, and this was later supported by Woolliams et al (1982),who showed that Scotish Blackface and Welsh Mountain ewes differed significantly in their plasma Cu levels. They also reported a breed differences in the efficiency of absorption of Cu in liver.

Results in the present study confirmed the protective effect of Zn supplementation against Cu toxicosis and the antagonism between Cu and Zn as shown by Bremner et al (1976) and Parry et al (1984).

Zn-binding to alpha2-M incubated with 150 μg Zn was significantly ($P<0.001$) lower in all three breeds as shown in Tables XXIII-XXVIII compared to alpha2-M incubated with 1500 μg Zn. However, the Zn-binding response of pregnant Dorset ewes (Table-XXIII) was different to Clun (Table-XXV) and Finn ewes (Table-XXVII) on Day-0 at level-I Zn incubation (150 μg Zn). Only 17.97 μg Zn/100 μg alpha2-M was bound compared to 69.90 and 31.38 μg Zn bound/100 μg alpha2-M in clun and Finn ewes respectively.

In Tables-XXIV, XXVI & XXVIII Zn-binding to alpha2-M incubated with 1500 μg Zn showed breed differences on Day-0. Clun ewes (Table-XXVI) showed significantly ($P<0.001$) greater Zn-binding than either Dorset or Finn ewes. For a difference of 10 times Zn (between level-II &

level-I) incubated with alpha2-M 16,7 and 11 times Zn was bound to alpha2-M in Dorset, Clun and Finn ewes respectively.

The existence of breed differences was clearly shown by Zn-binding to alpha2-M at both levels of Zn incubation. Dorset ewes showed more Zn-binding to alpha2-M at both levels of Zn (150 and 1500 μg Zn) incubation (Tables-XXIII & XXIV) than either Clun (Tables-XXV & XXVI) or Finn (Tables-XXVII & XXVIII) ewes. Finn ewes showed least Zn-binding among the three breeds and Zn-binding to alpha2-M reflected variations in plasma concentrations.

In vitro Zn-binding to alpha2-M responded to variations in plasma Cu suggested the dependence on the rate of Cu accumulation in the liver. In this study (Expt.7) the proportion of Zn bound to alpha2-M and albumin pool were not determined (due to lack of sufficient plasma from the experimental samples) which could have helped in explaining whether any transfer of Zn in vivo has taken place between these two pools to influence Zn-binding to alpha2-M.

SECTION-IV

GENERAL DISCUSSION

The aim of this investigation was to make a quantitative assessment of Zn-binding to alpha2-M in sheep; no previous attempts are recorded. Dietary copper had some influence on this binding, but influence of the ratio of dietary Cu to Zn although inconclusive warrants further research.

The absence of methods for the purification of sheep plasma alpha2-M necessitated testing the methods used for human plasma alpha2-M. A great deal of the work was carried out in purification of alpha2-M from sheep plasma and the alpha2-M purification method published by Song et al (1975) was used. In contrast to Song's et al (1975) findings who reported three protein peaks were observed compared with only one peak for a similar human plasma sample. Peak I contained alpha2-M and detectable amounts of IgG. When alpha2-M was purified by immunoadsorbent chromatography IgG was a contaminant. This IgG did not show any evidence of Zn-binding (Appendix-I) and its presence was ignored in binding studies.

Support for the hypothesis that Cu influences the binding of Zn to alpha2-M was obtained by showing the influence

of *in vitro* Cu concentrations on Zn-binding to sheep alpha2-M. This was followed by the changes in Zn-binding to alpha2-M in the plasma of sheep fed a Cu-deficient and high-Cu diet. Finally, the influence of a diet containing variable amounts of Cu and Zn in the ratio 1:4 on Zn-binding to alpha2-M in the plasma from pregnant Dorset, Clun and Finn ewes maintained indoors was studied.

Sheep alpha2-M was found to bind large amounts of Zn; this was shown in Expt. 3. Zn-binding to alpha2-M increased in a linear fashion with each addition of Zn from 150 to 3000 μg to 100 μg alpha2-M (Table-V and Fig.28). It was shown that 325.44 x 10^2 g-atoms Zn/mol alpha2-M was bound *in vitro* when 3000 μg Zn was incubated with 100 μg alpha2-M (Expt. 3). When 50,100,150,200 and 250 μg alpha2-M were each incubated separately with 1500 μg Zn, both, mean ug Zn bound/100 μg alpha2-M and g-atoms Zn/mol alpha2-M showed a 96.4% reduction at 250 ug alpha2-M concentration compared to 50 μg alpha2-M (Expt. 2, Table-IV, Fig.27).

Before it was possible to test the influence of copper, either dietary related, or direct plasma copper increase, on the capacity of alpha2-M to bind Zn, it was necessary in Expt.2 to test whether there was a linear relationship between the amount of alpha2-M and its ability to bind a constant quantity of Zn. In Expt. 2, when added Zn

concentration was kept constant and alpha2-M concentration increased, it was shown that the expected relationship existed. There have been no previous reports of similar studies in sheep or humans.

Recently Gonias et al (1984), Pratt and Pizzo (1984) reported Zn-binding capacity in excess of 100 g-atoms Zn/mol of human alpha2-M . In this study, a higher Zn-binding capacity was observed.

Evidence from the current studies with sheep alpha2-M and results on human alpha2-M by Gonias et al (1984), Pratt and Pizzo (1984), and Adham et al (1977) gives rise to the hypothesis that alpha2-M could have a homeostatic and transport function, controlling limited variation in plasma Cu and Zn in response to fluctuating dietary levels of these elements. Evidence in support of the above hypothesis was demonstrated in the current experiments by using sheep plasma alpha2-M. Sheep alpha2-M showed 4.5 times greater Zn-binding affinity than sheep albumin (Table-XIV, Expt.5); in terms of g-atoms Zn/mol alpha2-M, the Zn content of sheep alpha2-M was 50 times higher than that of albumin. These values are far higher than human alpha2-M, where, 50% higher affinity for Zn and 20 times higher Zn content than albumin were observed by Adham et al (1977) after dialysis. This suggests that not only alpha2-M has a role in the transport of Zn, but that has increased binding

capacity is important.

The molar ratio of Zn to alpha2-M *in vitro* was shown to be greater than the *in vivo* ratios reported so far in human plasma. Thus, a maximum of 222.16 x 10^2 ; 325.44 x 10^2 ; 198.16 x 10^2 ; 360.46 x 10^3 ; 76.39 x 10^3 g-atoms Zn/mol alpha2-M were observed. The highest concentration of Zn bound to alpha2-M (360.46 x 10^3 g-atoms Zn/mol alpha2-M) was in Cu-deficient sheep. Similarly, in pregnant ewes maintained indoors on a variable Cu and Zn (ratio 1:4) diet, 118.49.10^3 ; 54.59.10^3 ; 38.71.10^3 g-atoms Zn/mol alpha2-M respectively were determined in Dorset, Clun and Finn ewes. There have been no similar studies in either human alpha2-M or in sheep alpha2-M. However, there are studies of Zn binding to native human plasma alpha2-M (Parisi and Vallee, 1970; Adham et al, 1977; Song and Adham, 1979) and in hyperzincaemic and hypozincaemic human sera (Song and Adham, 1979). In both hypo and hyperzincemic sera obtained from patients with multiple myeloma and lymphocytic lymphoma, Song and Adham (1979) showed that the molar ratio between Zn and alpha2-M was maintained at approximately 1:1.

An investigation of the influence of Cu on Zn-binding to sheep plasma alpha2-M showed that the Zn-binding to sheep plasma alpha2-M in the presence of both Cu and Zn (Table-VIII, Expt.4) was less effective than incubation with Cu only (Tables-VI & VII, Expt.4). The effectiveness

was judged on the basis of Zn bound to alpha2-M and the time taken to respond to the influence of Cu. A significant ($P<0.001$) decrease in Zn-binding to alpha2-M (Tables-VI & VII, Expt.4) was observed as a direct consequence of incubating alpha2-M first with Cu (5 to 25 μg). The results in Table-VIII showed comparatively less bound Zn and slower response as both Cu and Zn were probably competing for the same sites on the alpha2-M. These results (Table-VI & VII, Expt.4) suggested that Cu occupied the Zn-binding sites. Comparison of Tables-VI, VII & VIII indicated that the levels of *in vitro* Cu and Zn are important considerations in assessing Zn-binding to alpha2-M; also, the incubation time or the period of association with the competing metals Cu and Zn is important.

The concentration of Cu and Zn in the incubation mixture appeared to guide the influence on Zn-binding to alpha2-M. This was shown clearly in Tables-VI & VII (Expt.4), where alpha2-M incubated with 150 μg Zn and 1500 μg Zn, the Zn-binding to alpha2-M decreased by 55.8% and 42% respectively in response to *in vitro* Cu ranging from 5-25 μg. It appears that higher *in vitro* Zn (1500 μg Zn) reduced the influence of Cu. This conclusion is supported by the results in Table-V (Expt.3), where a linear relationship between a constant concentration of alpha2-M and increasing concentrations of Zn was established in the absence of copper.

Studies on human plasma alpha2-M established that there are two Zn-binding sites in human alpha2-M (Adham et al, 1977; Pratt and Pizzo, 1984; Gettins and Cunningham, 1986).

Sheep plasma alpha2-M may be similar to human plasma alpha2-M in possessing Zn-binding sites with different affinities. The results in Expts.2, 3 and 4 demonstrated this by showing altered Zn-binding to alpha2-M in the presence and absence of Cu. This suggests the possibility of the occupation of Zn-binding sites on alpha2-M by Cu ions. It can be speculated that sheep plasma alpha2-M might be similar to human alpha2-M and it is the weaker sites which might be susceptible to Cu.

Studies of human plasma alpha2-M (Gettins and Cunningham, 1986) indirectly suggested the possibility of replacement of Zn in alpha2-M by Cu. They obtained similar data with Zn and Mn binding to human apo-alpha2-M and established that Mn has similar relative affinities for the two types of sites as Zn. Ions such as Mn(II) and Cu(II) have long electron-spin relaxation times and are therefore good NMR probes (Dwek, 1973). Thus supporting indirectly the replacement of Zn by Cu. Experimental evidence for this is offered by the results in Expts.4 (Tables-VI & VII) and 6 (Tables-XVI & XVII).

Feeding a Cu-deficient diet significantly ($P<0.001$)

decreased plasma Cu and Zn concentrations (Table-IX). However, after 7 weeks mean Zn concentration reached nearly the same level observed at the beginning (Fig.31).This confirmed the results by Saylor and Leach (1980) and Al-Mukhtar (1984) who observed reduced plasma Cu concentrations in Cu-deficient sheep similar to the results in this study. While reduced mean plasma Cu concentration may be directly related to lack of dietary Cu, the reduction in plasma Zn was not so obvious and may have been due to interrelationships between Zn and Cu. There is evidence of redistribution of Zn from tissues to liver under stressful conditions (Vikbladh, 1951; Beisel and Pekarek, 1972; Pekarek and Wannemacher, 1972; Pekarek, 1972; Pekarek and Beisel, 1974. It is possible that the Cu-deficiency results in a redistribution of Zn from tissues to liver. This would explain the lower plasma Zn as observed in Table IX, Expt.5. However, greater affinity between alpha2-M and Zn and lack of Cu in the diet stimulated alpha2-M to bind Zn. Occurence of this event may be responsible for the increase in plasma Zn after 7 weeks as shown in Table-IX, Expt.5.

A high-Cu diet was fed to sheep in Expt.6 and observations made on plasma Zn and Cu concentrations as shown in Table-XV. The results showed a rise of plasma Cu after one week; the increased concentration of Cu although higher than normal for three weeks indicating some support for Saylor and Leach (1980) who found

similar results and proposed that such an increase in plasma Cu was due to a lack of biliary control for Cu in sheep. However, after the first week, the plasma Cu concentration decreased, thus indicating that the Cu is leaving the plasma more rapidly and probably accumulating in the liver. There is no evidence that the biliary control has changed during these weeks of decreasing plasma Cu; however, after the ninth week, two of the sheep died; on post mortem examination showed liver Cu concentrations of toxic levels.

The mean plasma Zn concentration in high-Cu sheep fell by 54% after 4 weeks and was 34.3% lower after 7 weeks compared to the mean concentration at the begining of the experiment (Expt. 6, Table-XV, Fig.36). The recovery of plasma Zn concentrations from their lowest concentrations may be due to 200 mg Zn/kg in the diet.

Comparison of results in Expt.6 with the results by Bremner et al (1976) highlights the influence of dietary high-Cu. Bremner et al (1976) showed that high dietary Zn effectively protects against Cu-toxicosis in sheep. Three dietary treatments were used in their studies, group A given the basal diet (29 mg Cu and 43 mg Zn/kg diet),groups B and C received additional supplements of Zn, made up to 220 and 420 mg Zn/kg diet respectively. After 9 weeks they found a 9% reduction in mean plasma Zn concentration in group A. In Expt. 6 (Table-XV) the

reduction in Zn concentration was more pronounced. Similarly, in their (Bremner et al, 1976) study mean plasma Cu concentrations in all treatment groups increased up to 16 weeks and showed 72%, 58.4%, 58.6% increase in A,B and C groups respectively. After which time all groups showed reduction in plasma Zn but the concentrations were higher than at the start of the experiment. The pronounced influence of high-Cu diet in this study may be due to 250 mg Cu/kg diet compared to 29 mg Cu/kg diet of Bremner et al (1976). The ratio of Cu to Zn in this study was 5:4 and in their diets, 29:43; 29:220 and 29:420 in groups A, B and C respectively. These differences in Cu:Zn and the mutual antagonism between these two were responsible in this study for more pronounced rise and fall in mean plasma Cu concentrations. Similarly, higher Zn concentrations in A,B and C diets of Bremner et al (1976) were responsible, not only for maintaining higher plasma Zn concentrations but also offered effective protection against Cu-toxicosis. However, the precise concentrations of Cu, Zn required to offer protection against Cu toxicity is still not clear and in addition to Zn the effects of other elements such as molybdenum and iron could also be important.

Despite the recognition of dietary antagonism (Smith and Larson, 1946) between Cu and Zn in the diet of rats, with the exception of Bremner et al (1976) dietary levels of

Zn were not specified in earlier studies. This makes comparisons of the present results in this thesis with other workers (Todd et al, 1962; Todd and Thompson, 1963; Ishmael et al, 1972) difficult.

Further evidence on the influence of Cu on Zn-binding to alpha2-M from Cu-deficient and high-Cu diet fed sheep was obtained in Expts.5 & 6. These results showed that Zn-binding to alpha2-M obtained from Cu-deficient sheep plasma increased (Tables-X & XI, Expt.5) significantly ($P<0.001$). In contrast, significantly ($P<0.001$) decreased Zn-binding was observed in alpha2-M obtained from high-Cu sheep plasma (Tables-XVI & XVII, Expt.6).

Zn-binding to alpha2-M obtained from Cu-deficient sheep plasma increased during 4 weeks, from 44.48 x 10^2 to 74.58 x 10^2 g-atoms Zn/mol alpha2-M when incubated with level-I Zn (150 μg) (Table-X, Expt.5). Maximum g-atoms Zn/mol alpha2-M was 124.52 x 10^2 observed after 3 weeks. After which time Zn-binding to alpha2-M varied. Zn-binding to alpha2-M showed an increase after 7 weeks when Cu was supplemented in the diet (5 mg Cu/kg and 50 mg Zn/kg diet). At 1500 μg Zn incubation Zn-binding to alpha2-M was significantly ($P<0.001$) higher than at the start of the experiment for 6 weeks (Table-XI, Expt.5). Level-II Zn concentration was 10 times higher than level-I but Zn-binding to alpha2-M was more than 10 times

that observed at level-I, this could be important evidence indicating existence of different binding sites and binding to different binding sites. At higher Zn concentrations during incubation, dissociation of alpha2-M might occur as observed under electrophoretic conditions (Pratt and Pizzo, 1984) and under such conditions Zn-binding sites may be available for occupation by Cu as shown in Expt.4.

Conformational changes in the alpha2-M molcule could have occured during incubation with 1500 µg Zn which may be responsible for increased Zn-binding observed for 6 weeks (Table-XI, Expt.5). The possibility of this could be supported by the studies of Adham et al (1977). Adham et al (1977) suggested that the occupation of second Zn-binding site (weaker affinity site) of alpha2-M molcule by Zn initiates irreversible conformational changes in the molecule that interferes with enzyme binding activity. It is likely that these conformational changes may be responsible for exposing the Zn-binding sites to bind more Zn.

It can be speculated that Zn-binding amino acids might have been released on incubation with 1500 µg Zn, which may be responsible for increased Zn-binding. Zn-binding to amino acids such as histidine, glutamine, threonine, cystine and lysine, increased ultrafiltrable65Zn (Prasad and Oberleas, 1970) when added to predialysed serum.

These Zn-binding amino acids are present in human alpha2-M (Heimburger et al, 1964; Dunn and Spiro, 1967; Bourrillon and Razafimahaleo, 1972) and glutamine as glutamic acid accounts for about 12.3 to 13.5% of alpha2-M. However,there are no reports of amino acid analysis of sheep alpha2-M. It is not certain whether Zn bound to these amino acids will account for all the additional Zn bound to alpha2-M.

Increased Zn-binding to alpha2-M observed in Cu-deficiency (Tables-X & XI, Expt.5) might have taken place at weaker Zn-binding sites in alpha2-M. Evidence already available (Parisi and Vallee, 1970) suggests that Zn in human alpha2-M is bound with a high stability constant, a characteristic feature of other metalloproteins (Vallee and Wacker, 1970). Despite maintaining increased Zn-binding at both levels of Zn (150 and 1500 μg) <u>in vitro</u> Zn-binding to alpha2-M showed variability. This indicates the binding of Zn has taken place at sites with low affinity for Zn. The existence of a class of site with greater affinity (Site 1) for Zn and a class of weaker site (Site 2) with low affinity for Zn was confirmed by Adham et al (1977), Gettins and Cunningham (1986).

In Expt. 6, Tables-XVI & XVII sheep fed on high-Cu diet showed significantly ($P<0.001$) reduced Zn-binding to alpha2-M than at the start of the experiment. The

Zn-binding decreased from 48 x 10^2 ($47.80.10^2$) to 24 x 10^2 ($24.12.10^2$) g-atoms Zn/mol alpha2-M at level-I Zn (150 μg) and from 76 x 10^3 ($76.39.10^3$) to 14 x 10^3 ($14.10.10^3$) g-atoms Zn/mol alpha2-M at level-II Zn (1500 μg) in 7 weeks. After one week on high-Cu diet, mean plasma Cu concentration increased from 0.73 μg/ml to 3.18 μg/ml. This rise in plasma Cu occured at the same time as a decrease of 86% in Zn-binding to alpha2-M at level-I and by 90.8% at level-II Zn incubation (Tables-XVI & XVII, Expt.6) after 1 and 2 weeks respectively. However, the trend reversed after reaching lowest values of Zn-binding to alpha2-M and this corresponded with decrease in plasma Cu concentrations. After 6 weeks on high-Cu diet the above relationship was not obvious. This could have been due to altered ratio of concentrations between Zn and Cu in plasma (Table-XV, Expt.6).

Despite showing significant reduction in Zn-binding to alpha2-M there was continuous variation <u>in vitro</u> Zn-binding probably reflecting changes in plasma Cu concentration. Zn-binding to alpha2-M reduced directly in response to <u>in vivo</u> plasma Cu was clearly demonstrated on examination of plasma Cu concentrations and Zn-binding to alpha2-M at both levels. The possibility of Zn-binding to different sites on alpha2-M was reflected in Zn-binding to alpha2-M (Table-XVI, week 1; Table-XVII, week 2). In Table-XVI the lowest Zn-binding was after one week, whereas, in Table-XVII the lowest Zn-binding was observed

after 2 weeks. Using the same alpha2-M samples demonstrated different binding properties indicating binding of Zn to different sites on alpha2-M. These results strongly suggested that Cu is responsible for the reduction in Zn-binding to alpha2-M and that the dietary concentrations of Cu and Zn were contributing to this; there are however many questions which are not answered of the relationship between the Cu and Zn in their competition for binding sites of different affinity. The existence of sites with high and low affinity for Zn have been confirmed for human alpha2-M by Adham et al (1977), Pratt and Pizzo (1984), and Gettins and Cunningham (1986).

A model (Fig.40) is proposed for the distribution of Cu in sheep plasma and liver when high-Cu diet was fed to sheep. This model broadly explains the changes taking place in plasma Cu as a result of high dietary Cu and its influence on Zn-binding to alpha2-M. The changes taking place in plasma are based on the results from Tables-XV,XVI & XVII. The distribution of Cu in liver (shown in Fig.40) was based partly on the studies by other workers as quoted.

The evidence in support of the above model is as follows:

When high-Cu diet was fed, plasma Cu concentration increased significantly ($P<0.001$) as shown in Expt.6.

During the same period *in vitro* Zn-binding to alpha2-M significantly decreased (Tables-XVI & XVII). This *in vitro* Zn-binding to alpha2-M showed an inverse relationship with plasma Cu in high-Cu sheep. The plasma Cu in animals is rapidly removed from circulation by liver (Owen, 1980). In animals the copper metabolism is maintained by homeostatic regulation by biliary excretion (Owen, 1980). Thus the rate of Cu accumulation (Suttle and Mills, 1966a) in liver influences the Cu concentration in plasma. The Zn-binding to alpha2-M in plasma reflects the corresponding changes in plasma Cu concentration. It is already established that the accumulation of Cu in liver leads to metallothionein synthesis (Bremner, 1987). The results in Expt.6 have shown that after feeding Cu-supplemented diet Zn-binding to alpha2-M increased in response to the availability of Cu and Zn.

The model proposed summarizes the results in this study and gives possible explanation to the variations in Zn-binding to alpha2-M. It was shown by Saylor and Leach (1980) that in sheep there is no biliary control of Cu removal. Hence, the model showed no biliary removal of Cu from liver as shown in the model of Owen (1980). Owen (1980) proposed a three compartment model which appears to explain liver Cu metabolism in general.

Further evidence which strongly demonstrated that Cu did

replace Zn on alpha2-M was obtained from the analysis of purified alpha2-M obtained from the plasma of high-Cu sheep as shown in Table-XIX,Expt.6. These results showed the presence of Cu in alpha2-M and increased in concentration from 0.04 to 0.4 mg/g alpha2-M (Table-XIX, Expt.6). Whereas, no Cu was detected in alpha2-M obtained from the plasma of Cu-deficient sheep (Table-XIII, Expt.5).

The concentration of Zn in purified alpha2-M from high-Cu sheep varied from 0.440 mg/g alpha2-M at week-0 to 1.330 mg/g alpha2-M during weeks-4 to 6. However, in Cu-deficient sheep as high as 10.75 mg Zn/g alpha2-M was observed. These in vivo concentrations of Zn in alpha2-M confirmed the in vitro observations, which showed significantly ($P<0.001$) increased Zn-binding in Cu-deficienct and significantly ($P<0.001$) decreased Zn-binding to alpha2-M in high dietary Cu sheep.

The severity of the influence of high-Cu diet was probably reduced by the dietary Zn concentration of 200 mg Zn/kg as suggested by Bremner et al (1976). The recommended dietary requirement for sheep (The Nutrition Requirements of Livestock, 1980) is 35-50 mg Zn/kg diet. However, in high-Cu diet, 200 mg Zn/kg diet was included to reduce the severity and to facilitate studying Zn-binding to alpha2-M.

Alpha2-M and albumin pools have been characterized as major non-exchangeable and exchangeable (loosely bound) Zn pools respectively for human serum (Vikbladh, 1951; Parisi and Vallee, 1970). In Cu-deficient (Expts.5) and high-Cu (Expt.6) sheep plasma interesting results were observed on the Zn concentration of alpha2-M pool (Pool-I) and albumin pool (Pool-II). In the Cu-deficient sheep, initially, the Zn concentration of Pool-II was higher than Pool-I and from week 3, Pool-I Zn was higher than Pool-II Zn from 3 to 9 weeks. These results showed two points: (a) that dietary Cu-deficiency affects albumin first with greater reduction of albumin bound Zn than alpha2-M bound Zn, which was shown by the results in Table-XI (Expt.5). Zn concentration of albumin pool decreased from week-0, (b) transfer of Zn occured between albumin and alpha2-M such that, alpha2-M pool of Zn increased.

There are no previously published reports of similar observations in Cu-deficient sheep. However, in Zn-deficient sheep, release of albumin bound Zn was suggested (Parry, 1976) which could maintain the unbound and physiologically available Zn in plasma. In human sera considerable variation in albumin bound Zn was reported by Foote and Delves (1984). They suggested that human albumin exhibits excessive Zn-binding capacity, but under normal physiological conditions Zn occupies less than

0.2% of the capacity. According to these workers, reduction in serum albumin concentration as observed in gastrointestinal and hepatic disease, reflects Zn-deficiency. This is due to the dependence of albumin synthesis on the availability of Zn (Bates and McClain, 1981). Hence, it is apparent that any variations in serum Zn may be due to variations in the concentration of albumin. However, no reports are available in sheep and particularly in Cu-deficient sheep. Further research is needed to confirm these suggestions in both human plasma as well as sheep plasma.

In Expt.5 the two protein pools (alpha2-M and albumin) there is an indication of increased Zn in Pool-I while Pool-II Zn decreased. It can only be assumed that albumin which binds Zn loosely, lost some of its Zn. Cu-deficiency probably decreased Cu bound to albumin. There was no indication of Zn replacing Cu. On the other hand Zn concentration of alpha2-M has been maintained higher than albumin. High affinity of alpha2-M greater than albumin (Adham et al, 1977; in this study Expt.5, Table-XIV & Fig.35) may be responsible for increased Zn in alpha2-M.

Results in Table-XIII, Expt.5 demonstrated that Zn-binding to alpha2-M increased <u>in vivo</u> confirming the results obtained from <u>in vitro</u> Zn-binding studies as shown in Tables-X & XI. Table-XIII showed upto 10.75 mg

Zn/g alpha2-M obtained from the plasma of Cu-deficient sheep. Thus Zn-binding to alpha2-M *in vivo* and *in vitro* showed similar trends.

Further evidence supporting *in vitro* and *in vivo* influence of Cu on Zn-binding to alpha2-M was obtained. For this, alpha2-M pool and albumin pool Zn concentrations from high-Cu sheep plasma were measured. Unlike, Cu-deficient sheep, Zn concentration of alpha2-M pool reduced. Despite Zn reduction in albumin pool, concentration of Zn in albumin pool remained higher than alpha2-M pool. Zn-bound to alpha2-M pool increased after 6 weeks, but Zn concentration at the begining was achieved only once. These results confirmed that in high-Cu sheep Zn-binding to alpha2-M decreased.

Zn-binding to alpha2-M (Tables-XXIII to XXVIII) in three breeds of pregnant ewes on a variable Cu and Zn diet with a ratio 1:4 supported the observations made in Cu-deficient and high-Cu sheep and confirmed the influence of dietary Cu. They further demonstrated that the influence of dietary Cu is breed dependent.

Dorset ewes showed increased Zn-binding to alpha2-M (Table-XXII & XXIII, Expt.7) for 74 days. Maximum Zn-binding was $193.78.10^{2}$ and $118.49.10^{3}$ g-atoms Zn/mol alpha2-M at level-I and level-II Zn incubation, respectively. This increased Zn-binding is similar to

binding observed in Cu-deficient sheep. However, Zn-binding to alpha2-M did not reach the maximum binding achieved in Cu-deficient sheep. This could have been due to Cu to Zn ratio (1:4) of the diet. Higher Zn concentration of diet offered protection against high Cu concentration due to antagonism between the two metals probably influencing the absorption of Cu at the intestine.

The results in this study supported the breed differences observed by Parry et al (1984) in pregnant Dorset, Clun and Finn ewes maintained indoors.

The three breeds (Expt.7) responded differently and showed mutual antagonism between Cu and Zn. Thus, the variable Cu, Zn (1:4) diet was responsible in Dorset for the signs of Cu-deficiency (increased Zn-binding to alpha2-M). In Clun and Finn ewes converse effect was true. The high-Cu conditon (Tables-XXIV to XXVII) of plasma was probably caused by lack of sufficient dietary Zn to antagonise Cu.

The effectiveness of the diet was judged by preventing death of ewes due to development of Cu-poisoning when kept indoors. Additional stress of pregnancy also existed. Though the diet was effective in preventing death it appears that the effect on Zn-binding to alpha2-M strongly suggests further evaluation of

protection offered and the basis of evaluation.

The evidence obtained from the results in this study support the hypothesis on the influence of dietary copper on Zn-binding to sheep plasma alpha2-M. The influence of copper was dependent on the ratio of dietary concentrations between Cu and Zn. The influence of Cu *in vivo* reflected the influence of the ratio between Cu and Zn in the diets.

The results in Expt.1 showed that sheep plasma alpha2-M can be purified using methods (Song et al, 1975; McEntire, 1978) used for human alpha2-M. Sheep plasma alpha2-M obtained from these methods showed that IgG is closely associated with alpha2-M. An isoelectric point of 4.8 was determined for sheep alpha2-M.

In Expts.2 & 3 the results showed that Zn-binding to alpha2-M is dependent on the concentration of Zn and a linear relationship between Zn-bound and alpha2-M.

In Expt.4 the results showed that Zn and Cu competed for the binding sites on alpha2-M and is influenced by the concentration of Cu and the incubation time with Cu and Zn.

In Expt.5, Zn-binding to sheep plasma alpha2-M increased both *in vivo* and *in vitro* in dietary Cu-deficiency.

In Expt.6, Zn-binding to plasma alpha2-M decreased both *in vivo* and *in vitro* in sheep on high-Cu diet. The presence of Cu in alpha2-M obtained from high-Cu sheep plasma strongly suggested that Cu occupied sites on alpha2-M probably replacing Zn.

In Expt.7 the results showed breed differences in Zn-binding to alpha2-M in pregnant Dorset, Clun and Finn ewes maintained indoors on a variable Cu, Zn diet with a ratio, 1:4.

The results in this thesis strongly suggest the influence of Cu on Zn-binding to alpha2-M and paved the way for the investigation of other Zn-binding proteins. The suceptibility of sheep to Cu toxicity enhances the importance of these studies by better understanding to improve productivity. However, further studies are needed to extend these studies to other breeds of sheep. These studies appeared to be more urgent to understand the mutual antagonism between Cu and Zn.

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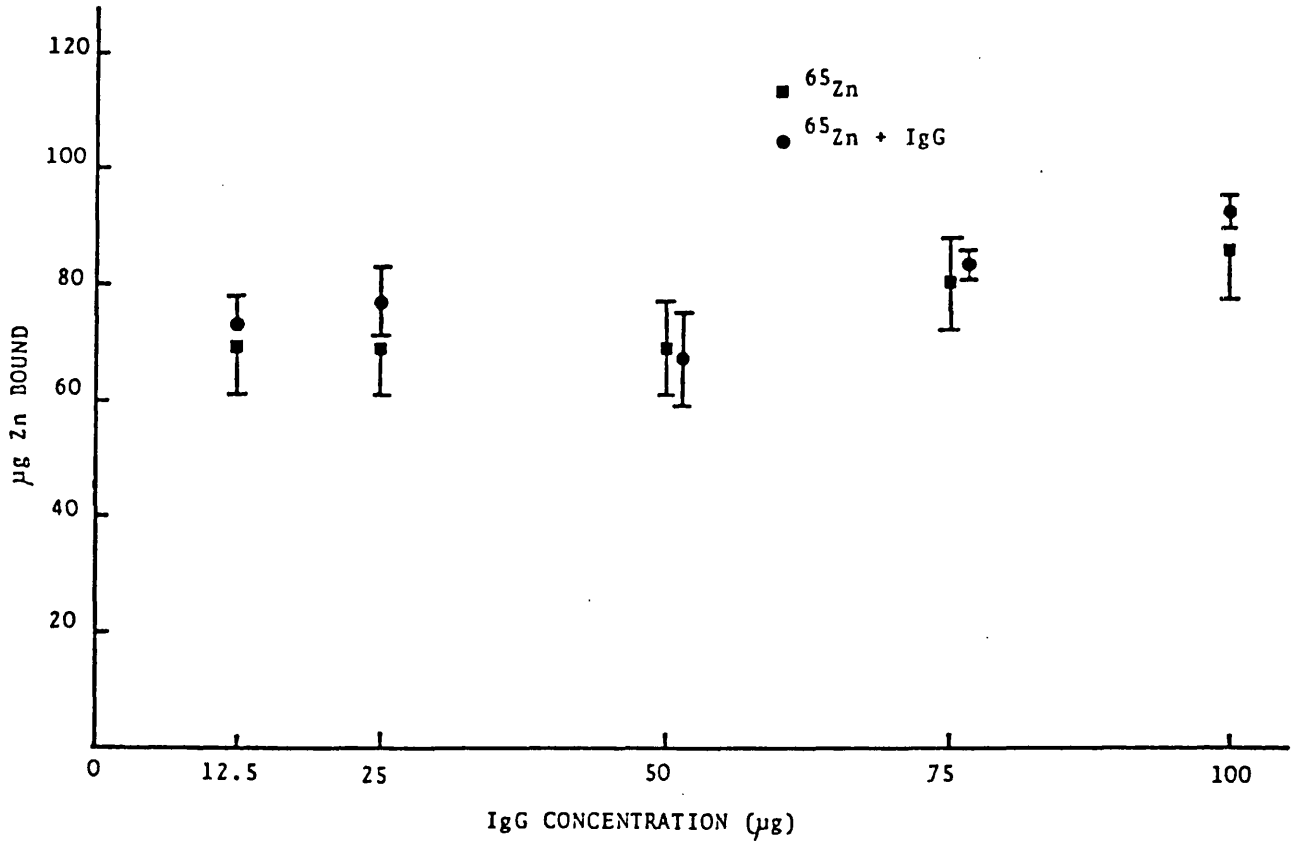
APPENDIX-I

Binding of ^{65}Zn to IgG and alpha2-M on PAGE

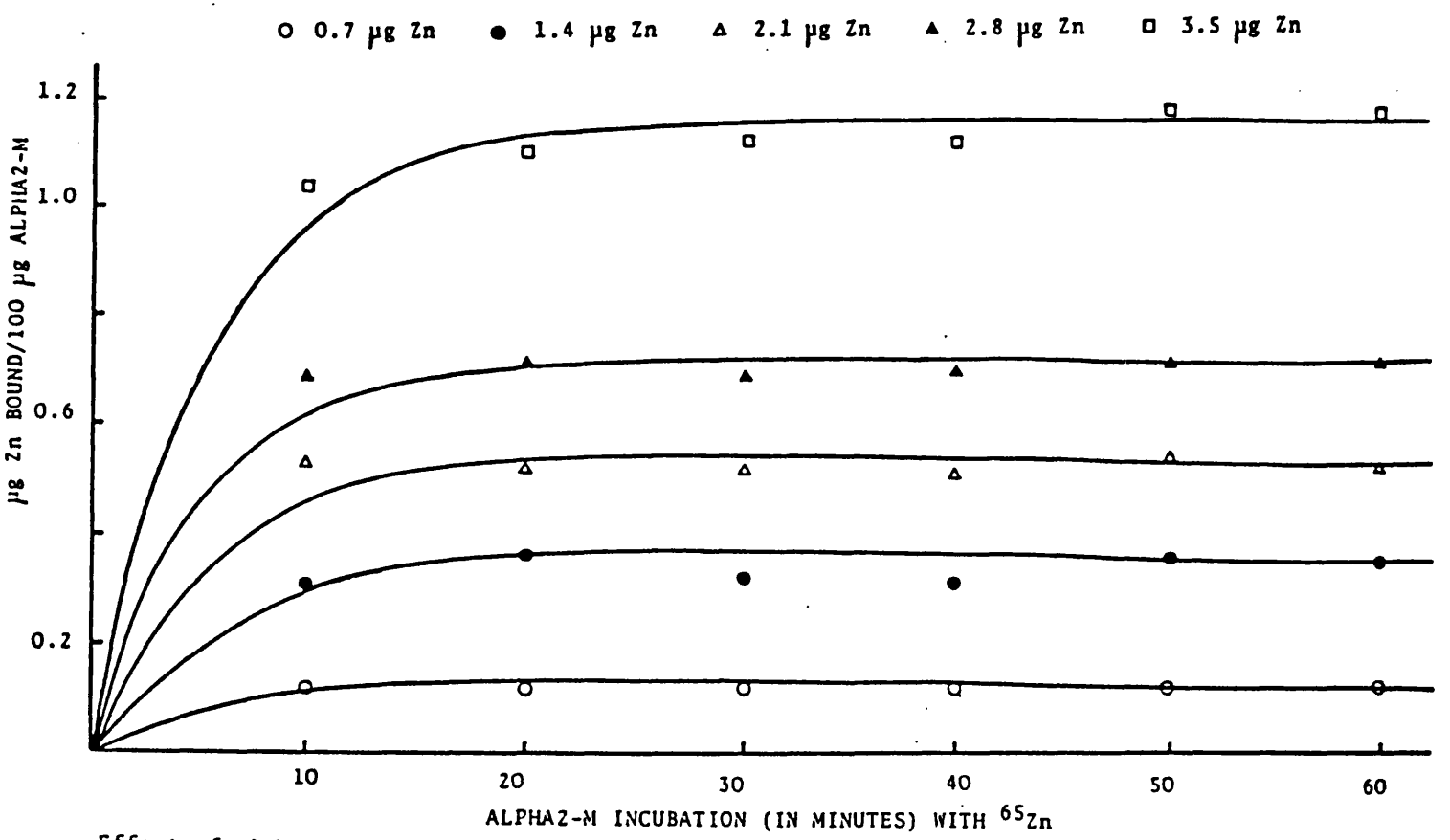
Sample applied (µl)	IgG (C.P.M.)	Alpha2-M (C.P.M.)	Blank (C.P.M.)
10	318	366	305
20	288	441	295
30	322	491	331
40	317	516	305
50	324	628	315
100	312	1069	295
200	348	1875	325

The above results showed that no ^{65}Zn is bound to IgG. ^{65}Zn is bound to only alpha2-M.

Results of ^{65}Zn binding to IgG using gel filtration technique is shown in Fig.47, which shows no ^{65}Zn binding to IgG compared to blank. Details of the experiment are given in Section-II,7.



Zn-binding to IgG at concentrations ranging from 12.5 µg to 100 µg on Sephadex G-200 column (details in Section-II,7).



Effect of alpha2-M incubation time (in minutes) with ^{65}Zn on Zn-binding to alpha2-M.

APPENDIX-III

Zn-binding to alpha2-M (50-250 ug) incubated with 1500 ug Zn

No.of assays	Concentration of alpha2-M (ug)				
	50	100	150	200	250
ROW	C1	C2	C3	C4	C5
	ug Zn Bound/100 ug alpha2-M				
1	181.50	31.80	33.10	24.22	8.34
2	193.72	58.50	13.60	18.59	7.55
3	210.56	46.31	25.72	12.95	6.18
4	250.64	39.71	19.85	15.66	5.90
5	163.30	45.35	23.58	19.70	8.21

```
MTB > TTEST C1 C2 C3 C4 C5

TEST OF MU =   0.0 VS MU N.E.   0.0
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	200.3	33.1	14.8	13.53	0.0002
C2	5	44.3	9.8	4.4	10.12	0.0005
C3	5	23.2	7.2	3.2	7.19	0.0020
C4	5	18.2	4.3	1.9	9.56	0.0007
C5	5	7.2	1.1	0.5	14.24	0.0001

APPENDIX-IV

Zn-binding to alpha2-M (100 ug) on varying incubated Zn from 150-3000 ug

No.of assays	CONCENTRATION OF Zn (ug) ADDED TO 100 ug ALPHA2-M					
	150	300	450	750	1500	3000
ROW	C1	C2	C3	C4	C5	C6
1	13.90	25.99	49.89	75.00	157.27	279.65
2	15.42	31.76	46.50	73.83	148.66	295.38
3	14.57	28.68	43.16	76.47	152.99	305.17
4	13.96	26.52	41.28	74.65	146.83	288.33
5	14.82	27.35	45.20	72.82	149.15	298.91

```
MTB > TTEST C1 C2 C3 C4 C5 C6

TEST OF MU =  0.000 VS MU N.E.  0.000
```

	μg Zn Bound/100 μg alpha2-M				
	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	14.454	0.739	0.331	43.71	0.0000
C2	5	28.060	2.304	1.030	27.23	0.0000
C3	5	45.206	3.287	1.470	30.75	0.0000
C4	5	74.554	1.361	0.609	122.48	0.0000
C5	5	150.900	4.125	1.845	81.79	0.0000
C6	5	293.408	9.789	4.378	67.02	0.0000

APPENDIX-V

Effect of in vitro Cu (5-25 ug) at level-I Zn

No.of ug Zn Bound/100 ug alpha2-M assays

ROW	C1	C2	C3	C4	C5
1	13.35	16.85	17.51	13.09	9.76
2	22.57	20.35	16.65	9.78	6.70
3	19.55	20.94	11.28	11.56	8.60
4	17.62	21.07	13.47	13.86	8.05
5	18.74	19.66	14.89	12.82	7.43

```
MTB > TTEST C1 C2 C3 C4 C5 C
```

```
TEST OF MU = 0.00 VS MU N.E. 0.00
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	18.37	3.35	1.50	12.25	0.0003
C2	5	19.77	1.73	0.77	25.60	0.0000
C3	5	14.76	2.50	1.12	13.22	0.0002
C4	5	12.16	1.57	0.70	17.28	0.0001
C5	5	8.11	1.16	0.52	15.58	0.0001

Effect of in vitro Cu (5-25 ug) at level-II Zn

No.of ug Zn Bound/100 ug alpha2-M assays

ROW	C6	C7	C8	C9	C10
1	173.52	151.02	111.12	127.48	93.24
2	169.81	161.07	96.35	128.67	102.75
3	169.79	169.80	126.25	136.21	105.14
4	171.05	158.34	118.71	122.85	98.68
5	168.56	154.65	114.92	119.66	95.08

```
MTB > TTEST C6 C7 C8 C9 C10
```

```
TEST OF MU = 0.000 VS MU N.E. 0.000
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C6	5	170.546	1.881	0.841	202.71	0.0000
C7	5	158.976	7.141	3.193	49.78	0.0000
C8	5	113.470	11.085	4.957	22.89	0.0000
C9	5	126.974	6.304	2.819	45.04	0.0000
C10	5	98.978	5.010	2.241	44.17	0.0000

APPENDIX-VI

No.of sheep — **Plasma Zn concentrations of Cu-deficient sheep**

ROW	C1	C2	C3	C4	C5	C6	C7	C8	C9
	Week 0	1	2	3	4	5	6	7	8
1	1.10	0.750	1.00	1.150	1.05	0.925	1.300	1.275	0.90
2	1.40	0.900	0.85	1.050	0.95	0.850	0.925	1.100	0.70
3	0.95	1.150	0.75	0.925	0.90	1.000	1.050	1.150	0.85
4	1.32	0.775	0.90	0.950	0.85	0.850	0.800	1.075	0.70
5	0.95	0.950	0.75	0.650	0.60	0.625	0.850	1.000	0.65

C10 = Week = 9

C10
0.925
0.925
0.800
0.850
0.925

C1 = Week-0
C2-C8 = Cu-deficient
C9 & C10 = Cu-suppl.

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU = 0.0000 VS MU N.E. 0.0000
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	1.1440	0.2084	0.0932	12.27	0.0003
C2	5	0.9050	0.1605	0.0718	12.61	0.0002
C3	5	0.8500	0.1061	0.0474	17.92	0.0001
C4	5	0.9450	0.1874	0.0838	11.27	0.0004
C5	5	0.8700	0.1681	0.0752	11.57	0.0003
C6	5	0.8500	0.1403	0.0627	13.55	0.0002
C7	5	0.9850	0.1997	0.0893	11.03	0.0004
C8	5	1.1200	0.1022	0.0457	24.51	0.0000
C9	5	0.7600	0.1084	0.0485	15.68	0.0001
C10	5	0.8850	0.0576	0.0257	34.38	0.0000

No.of sheep — **Plasma Cu concentrations of Cu-deficient sheep**

ROW	C11	C12	C13	C14	C15	C16	C17	C18	C19
	Week 0	1	2	3	4	5	6	7	8
1	0.700	0.450	0.525	0.625	0.550	0.550	0.575	0.650	0.550
2	1.050	0.650	0.850	0.925	0.775	0.750	0.975	1.025	0.900
3	1.075	0.750	0.750	0.825	0.775	0.800	0.825	0.825	0.800
4	1.825	0.825	1.000	0.925	0.825	0.875	1.000	1.025	0.950
5	1.300	1.025	0.850	0.750	0.650	0.725	0.800	0.900	1.075

C20 = Week = 9

C20
0.775
0.850
0.650
0.900
0.950

C11 = Week-0
C12-C18 = Cu-deficient
C19 & C20 = Cu-suppl.

```
MTB > TTEST C11 C12 C13 C14 C15 C16 C17 C18 C19 C20

TEST OF MU = 0.000 VS MU N.E. 0.000
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C11	5	1.190	0.415	0.186	6.41	0.0030
C12	5	0.740	0.213	0.095	7.78	0.0015
C13	5	0.795	0.175	0.078	10.14	0.0005
C14	5	0.810	0.127	0.057	14.26	0.0001
C15	5	0.715	0.113	0.050	14.19	0.0001
C16	5	0.740	0.121	0.054	13.71	0.0002
C17	5	0.835	0.170	0.076	10.98	0.0004
C18	5	0.885	0.157	0.070	12.63	0.0002
C19	5	0.855	0.197	0.088	9.70	0.0006
C20	5	0.825	0.117	0.052	15.73	0.0001

APPENDIX-VII

Zn-binding to alpha2-M in Cu-deficient sheep plasma at level-I Zn

μg Zn BOUND/100 μg alpha2-M

```
MTB > PRINT C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11
```

ROW	C1	C2	C3	C4	C5	C6	C7	C8
Week	0	1	2	3	4	5	6	7
1	39.11	34.14	69.13	112.28	68.83	17.56	44.29	7.53
2	41.58	40.25	64.59	108.42	72.56	19.27	50.14	10.65
3	35.65	30.42	76.92	116.65	76.47	22.38	39.85	14.36
4	45.56	37.44	71.26	102.66	54.98	14.54	46.90	9.77
5	38.60	32.68	67.87	121.35	63.45	16.82	38.94	7.95

ROW	C9	C10
8		9
1	54.78	50.51
2	59.35	64.34
3	48.84	46.25
4	51.60	48.75
5	60.55	49.38

No.of assays = 5

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11

TEST OF MU =  0.00 VS MU N.E.  0.00
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	40.10	3.71	1.66	24.17	0.0000
C2	5	34.99	3.89	1.74	20.10	0.0000
C3	5	69.95	4.58	2.05	34.13	0.0000
C4	5	112.27	7.23	3.23	34.75	0.0000
C5	5	67.26	8.38	3.75	17.95	0.0001
C6	5	18.11	2.93	1.31	13.83	0.0002
C7	5	44.02	4.72	2.11	20.87	0.0000
C8	5	10.05	2.73	1.22	8.24	0.0012
C9	5	55.02	4.98	2.23	24.70	0.0000
C10	5	51.85	7.16	3.20	16.20	0.0001

Zn-binding to alpha2-M in Cu-deficient sheep plasma at level-II Zn

μg Zn BOUND/100 μg alpha2-M

ROW	C1	C2	C3	C4	C5	C6	C7	C8
Week	0	1	2	3	4	5	6	7
1	347.44	469.03	979.21	3255.99	811.88	706.29	1866.47	199.84
2	368.52	503.85	899.62	3518.32	750.56	747.62	2005.22	250.63
3	305.65	485.66	941.77	3168.70	867.37	686.57	1792.75	176.85
4	372.98	405.28	1008.45	3095.51	921.74	712.93	1812.66	159.76
5	351.31	418.74	1048.60	3210.86	786.65	696.72	1935.25	221.82

ROW	C9	C10
Week	8	9
1	1121.74	402.38
2	1086.55	295.56
3	1179.35	365.70
4	1099.65	418.43
5	1105.47	408.67

No.of assays =5

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU =  0.0 VS MU N.E.  0.0
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	349.2	26.7	11.9	29.29	0.0000
C2	5	456.5	42.7	19.1	23.90	0.0000
C3	5	974.7	59.1	26.4	36.90	0.0000
C4	5	3249.9	161.3	72.1	45.06	0.0000
C5	5	827.6	67.6	30.3	27.36	0.0000
C6	5	710.0	23.3	10.4	68.28	0.0000
C7	5	1882.5	88.1	39.4	47.79	0.0000
C8	5	201.8	36.0	16.1	12.54	0.0002
C9	5	1118.6	36.3	16.2	68.98	0.0000
C10	5	378.1	50.3	22.5	16.81	0.0001

APPENDIX-VIII

No.of sheep — Plasma Zn concentrations of high-Cu sheep

ROW	C1 Week 0	C2 1	C3 2	C4 3	C5 4	C6 5	C7 6	C8 7	C9 8
1	2.375	1.625	1.500	1.275	0.875	1.25	1.750	1.300	1.35
2	2.000	1.475	1.470	1.050	0.950	1.55	1.325	0.950	1.12
3	2.500	1.675	1.525	1.105	1.000	1.40	1.175	1.450	1.40
4	1.750	1.700	1.375	1.125	0.925	1.65	1.400	1.650	1.30
5	1.700	1.645	1.475	1.175	1.075	1.15	1.125	1.575	1.45

C10 = Week 9

C10
1.00
2.25
1.30
1.20
1.00

C1 = Week 0
C2-C8 = high-Cu
C9 & C10 = Cu-suppl.

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU = 0.000 VS MU N.E. 0.000
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	2.105	0.412	0.184	11.43	0.0003
C2	5	1.624	0.088	0.039	41.24	0.0000

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C3	5	1.469	0.057	0.025	57.69	0.0000
C4	5	1.146	0.085	0.038	30.19	0.0000
C5	5	0.965	0.076	0.034	28.30	0.0000
C6	5	1.400	0.206	0.092	15.19	0.0001
C7	5	1.355	0.247	0.111	12.26	0.0003
C8	5	1.385	0.277	0.124	11.18	0.0004
C9	5	1.324	0.127	0.057	23.31	0.0000
C10	5	1.350	0.520	0.232	5.81	0.0044

No.of sheep — Plasma Cu concoentration of high-Cu sheep

ROW	C1 Week 0	C2 1	C3 2	C4 3	C5 4	C6 5	C7 6	C8 7	C9 8	C10 9
1	0.825	3.65	1.825	1.15	1.425	1.10	0.2	0.100	0	0
2	0.800	3.50	1.750	1.35	1.125	1.10	0.2	0.000	0	0
3	0.650	2.55	1.650	0.90	0.950	0.80	0.2	0.100	0	0
4	0.725	3.80	1.900	0.90	0.800	0.95	0.0	0.100	0	0
5	0.650	2.40	1.150	0.90	0.650	0.70	0.0	0.125	0	0

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU = 0.0000 VS MU N.E. 0.0000
```

C1 = Week-0
C2-C8 = high-Cu
C9 & C10 = Cu-suppl.

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	0.7300	0.0818	0.0366	19.96	0.0000
C2	5	3.1800	0.6544	0.2927	10.87	0.0004
C3	5	1.6550	0.2971	0.1329	12.46	0.0002
C4	5	1.0400	0.2043	0.0914	11.38	0.0003
C5	5	0.9900	0.3003	0.1343	7.37	0.0018
C6	5	0.9300	0.1789	0.0800	11.63	0.0003
C7	5	0.1200	0.1095	0.0490	2.45	0.070
C8	5	0.0850	0.0487	0.0218	3.90	0.018

APPENDIX-IX

Zn-binding to alpha2-M in high-Cu sheep plasma at level-I Zn

µg Zn BOUND/100 µg alpha2-M

ROW	C1	C2	C3	C4	C5	C6	C7	C8	C9
Week	0	1	2	3	4	5	6	7	8
1	19.07	5.36	10.45	19.88	27.38	39.33	41.92	19.82	29.55
2	53.03	6.80	15.92	18.65	29.45	38.65	40.80	25.96	24.33
3	44.66	4.90	8.65	25.32	26.84	45.22	43.46	21.55	35.65
4	38.45	7.58	7.55	16.79	24.57	36.59	47.22	24.69	30.27
5	60.28	5.25	10.16	15.86	27.66	38.71	36.78	16.72	28.85

C10 (Week 9)

75.11	80.26	67.95	71.45	68.54

No.of assays = 5

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU =  0.00 VS MU N.E.  0.00
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	43.10	15.77	7.05	6.11	0.0036
C2	5	5.98	1.15	0.52	11.60	0.0003
C3	5	10.55	3.23	1.44	7.31	0.0019
C4	5	19.30	3.71	1.66	11.62	0.0003
C5	5	27.18	1.76	0.79	34.59	0.0000
C6	5	39.70	3.25	1.46	27.28	0.0000
C7	5	42.04	3.81	1.70	24.67	0.0000
C8	5	21.75	3.72	1.66	13.06	0.0002
C9	5	29.73	4.04	1.81	16.45	0.0001
C10	5	72.66	5.11	2.28	31.81	0.0000

Zn-binding to alpha2-M in high-Cu sheep plasma at level-II Zn

µg Zn BOUND/100 µg alpha2-M

ROW	C1	C2	C3	C4	C5	C6	C7	C8
Week	0	1	2	3	4	5	6	7
1	983.46	456.69	65.22	214.03	287.91	447.44	452.44	126.52
2	774.68	302.73	74.69	175.88	312.66	468.39	439.62	149.11
3	657.59	397.45	68.36	150.67	276.54	398.65	521.75	131.78
4	728.82	442.38	59.52	225.46	255.39	507.13	476.77	109.67
5	698.74	476.95	49.85	186.43	291.10	435.81	416.55	118.22

ROW	C9	C10
	8	9
1	196.62	683.57
2	172.44	720.28
3	215.79	650.16
4	230.65	641.55
5	168.75	682.47

No. of assays = 5

```
MTB > TTEST C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

TEST OF MU =  0.0 VS MU N.E.  0.0
```

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	688.7	72.7	32.5	21.18	0.0000
C2	5	455.2	37.5	17.7	25.79	0.0000
C3	5	63.5	9.4	4.2	15.10	0.0001
C4	5	190.5	30.0	13.4	14.21	0.0001
C5	5	284.7	21.0	9.4	30.36	0.0000
C6	5	451.5	40.1	17.9	25.17	0.0000
C7	5	461.4	40.1	18.0	25.71	0.0000
C8	5	127.1	14.9	6.7	19.05	0.0000
C9	5	196.8	26.9	12.0	16.39	0.0001
C10	5	675.6	31.3	14.0	48.30	0.0000

APPENDIX-X

Effect of varying dietary Cu and Zn concentrations in the ratio 1:4 on Zn-binding to alpha2-M in the plasma of pregnant Dorset ewes maintained indoors

Alpha2-M incubated with level-I Zn (150 ug)

ROW	C1	C2	C3	C4	C5	C6	C7
	Days 0	35	42	55	69	74	86
1	8.90	149.32	37.72	36.30	75.05	60.75	17.07
2	19.59	212.66	108.99	43.99	85.94	28.50	3.30
3	25.32	178.94	75.68	51.29	63.72	49.66	18.69
4	12.65	163.72	69.88	46.26	71.97	52.78	25.68
5	23.40	172.91	76.45	44.53	68.45	58.20	19.25

MTB > TTEST C1 C2 C3 C4 C5 C6 C7 Days = Days on experimental diet

TEST OF MU = 0.00 VS MU N.E. 0.00
ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	17.97	7.01	3.14	5.73	0.0046
C2	5	174.71	24.73	11.06	15.80	0.0001
C3	5	73.74	25.33	11.33	6.51	0.0029
C4	5	44.39	5.41	2.42	18.33	0.0001
C5	5	72.87	8.22	3.68	19.82	0.0000
C6	5	49.98	12.78	5.71	8.75	0.0009
C7	5	16.80	8.23	3.68	4.57	0.010

Alpha2-M incubated with level-II Zn (1500 ug)

ROW	C1	C2	C3	C4	C5	C6	C7
	Days 0	35	42	55	69	74	86
1	267.02	1189.72	677.19	639.65	910.43	404.96	331.04
2	333.12	952.18	607.79	488.67	907.04	292.20	198.75
3	284.69	1215.35	728.44	528.25	842.64	375.95	227.46
4	252.75	1086.96	621.73	437.76	815.25	328.72	202.93
5	290.58	897.45	598.86	490.45	795.66	416.17	210.07

MTB > TTEST C1 C2 C3 C4 C5 C6 C7 Days=Days on experimental diet

TEST OF MU = 0.0 VS MU N.E. 0.0
ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	285.6	30.4	13.6	20.98	0.0000
C2	5	1068.3	140.9	63.0	16.96	0.0001
C3	5	646.8	54.9	24.5	26.36	0.0000
C4	5	517.0	75.8	33.9	15.26	0.0001
C5	5	854.2	52.5	23.5	36.37	0.0000
C6	5	363.6	52.3	23.4	15.55	0.0001
C7	5	234.0	55.3	24.7	9.46	0.0007

APPENDIX-XI

Effect of varying dietary Cu and Zn concentrations in the ratio 1:4 on Zn-binding to alpha2-M in the plasma of pregnant Clun ewes maintained indoors

Alpha2-M incubated with level-I Zn (150 ug)

ROW	Days C1 0	C2 14	C3 21	C4 35	C5 40	C6 48	C7 62
1	37.94	24.81	23.68	26.90	7.60	11.74	27.01
2	121.92	21.47	24.37	17.99	12.25	8.63	7.17
3	55.00	23.17	26.72	21.35	9.95	10.27	14.15
4	69.75	27.91	22.54	19.87	8.74	7.55	8.93
5	64.83	25.56	24.85	18.66	11.48	9.76	11.47

MTB > TTEST C1 C2 C3 C4 C5 C6 C7 Days=Days on experimental diet

TEST OF MU = 0.0 VS MU N.E. 0.0 ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	69.9	31.5	14.1	4.96	0.0077
C2	5	24.6	2.4	1.1	22.55	0.0000
C3	5	24.4	1.5	0.7	35.34	0.0000
C4	5	21.0	3.6	1.6	13.16	0.0002
C5	5	10.0	1.9	0.9	11.71	0.0003
C6	5	9.6	1.6	0.7	13.43	0.0002
C7	5	14.1	8.7	3.9	3.63	0.022

Alpha2-M incubated with level-II Zn (1500 ug)

ROW	Days C8 0	C9 14	C10 21	C11 35	C12 40	C13 48	C14 62
1	723.05	349.87	409.71	416.04	104.91	152.97	277.05
2	363.05	317.95	402.72	404.35	145.59	171.41	375.86
3	412.74	356.22	421.76	388.36	128.45	180.38	225.50
4	563.67	402.55	385.63	418.95	107.19	141.56	312.78
5	398.38	376.00	391.84	398.51	116.21	157.45	305.82

MTB > TTEST C8 C9 C10 C11 C12 C13 C14 Days=Days on experimental diet

TEST OF MU = 0.0 VS MU N.E. 0.0 ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C8	5	492.2	150.2	67.2	7.33	0.0019
C9	5	360.5	31.4	14.1	25.65	0.0000
C10	5	402.3	14.3	6.4	62.79	0.0000
C11	5	405.2	12.6	5.6	71.88	0.0000
C12	5	120.5	16.8	7.5	16.02	0.0001
C13	5	160.8	15.2	6.8	23.67	0.0000
C14	5	313.8	36.9	16.5	18.99	0.0000

APPENDIX-XII

Effect of varying dietary Cu and Zn concentrations in the ratio 1:4 on Zn-binding to alpha2-M in the plasma of pregnant Finn ewes maintained indoors

Alpha2-M incubated with level-I Zn (150 ug)

ROW	C1	C2	C3	C4	C5
	Days 0	14	40	48	62
1	38.95	9.77	24.23	2.27	20.48
2	41.22	9.14	18.47	5.76	20.77
3	24.76	12.05	22.68	11.88	16.09
4	20.55	7.96	17.59	7.50	23.14
5	31.43	18.87	20.35	9.66	19.17

MTB > TTEST C1 C2 C3 C4 C5 Days=Days on experimental diet

TEST OF MU = 0.00 VS MU N.E. 0.00

ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C1	5	31.38	8.88	3.97	7.90	0.0014
C2	5	11.56	4.35	1.95	5.94	0.0040
C3	5	20.66	2.79	1.25	16.56	0.0001
C4	5	7.41	3.68	1.65	4.50	0.011
C5	5	19.93	2.58	1.15	17.27	0.0001

Alpha2-M incubated with level-II Zn (1500 ug)

ROW	C6	C7	C8	C9	C10
	Days 0	14	40	48	62
1	402.77	89.35	168.07	59.00	184.54
2	321.49	103.22	165.08	104.30	163.70
3	295.65	81.91	154.67	68.75	202.35
4	346.74	112.76	179.23	85.87	171.57
5	378.22	79.51	146.35	91.20	180.83

MTB > TTEST C6 C7 C8 C9 C10 Days=Days on experimental diet

TEST OF MU = 0.0 VS MU N.E. 0.0

ug Zn Bound/100 ug alpha2-M

	N	MEAN	STDEV	SE MEAN	T	P VALUE
C6	5	349.0	42.9	19.2	18.20	0.0001
C7	5	93.3	14.3	6.4	14.65	0.0001
C8	5	162.7	12.6	5.7	28.77	0.0000
C9	5	81.8	18.0	8.1	10.14	0.0005
C10	5	180.6	14.6	6.5	27.61	0.0000

only 250 mg Fe/kg. It appears therefore that the rats adapted in some way to the highest level of Fe supplementation, and this may explain why only minor effects on Cu availability were observed previously when rats were given a diet with 1000 mg Fe/kg for 11 weeks (Bremner & Young, 1981).

Expt. 2.

Further evidence of a rapid reduction in the apparent availability of Cu in Fe-treated rats was obtained using the technique of Price & Chesters (1985). In this procedure the degree of restoration in intestinal cytochrome oxidase activity which occurs when Cu-deficient rats are repleted with Cu is used as a measure of Cu availability. When cytochrome oxidase activity was calculated relative to that of NADH cytochrome c reductase, values of 0.83 ± 0.11 were obtained in the untreated Cu-deficient rats, whereas those in rats repleted with 10 μgCu/d and given 50, 250 or 500 mg Fe/kg diet were 1.70 ± 0.12, 1.26 ± 0.11 and 0.96 ± 0.10 respectively. This indicates that the availability of Cu was reduced by 50 and 85% within only 4 days by dietary Fe contents of 250 and 500 mg/kg respectively.

Fe is therefore a more potent antagonist of Cu metabolism than has hitherto been suspected. The mechanism(s) whereby Fe has this effect is unknown but, in rats at least, would appear not to involve inhibition of intestinal Cu absorption (Bremner & Young, 1981). Animals appear to adapt to continued intakes of large amounts of Fe and the effects of Cu metabolism diminish in intensity, with resultant development of abnormal dose-response relationships.

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Effects of High Dietary Zinc on Copper Transport in Three Breeds of Housed Pregnant Sheep

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There is very little evidence that a dietary level of Cu can be recommended for sheep under the stress of housing and pregnancy which lessens the risk of Cu toxicity. Although evidence by Bremner *et al.* (1976) indicated that increased dietary Zn in housed ewe lambs functioned as a metabolic antagonist, Campbell & Mills (1979) found that dietary Zn supplementation in pregnant ewes resulted in symptoms of Cu deficiency and Zn toxicity. Such evidence would suggest that the ratio of Zn:Cu could be more important than the absolute amounts.

This study set out to test whether differences could be observed in the plasma bound Cu and Zn in three breeds of housed pregnant sheep fed a Zn-supplemented diet.

I. Experimental design and Methods

Thirty-six pregnant ewes were allocated to three groups of different breeds - Dorset, Clun and Finn, each of twelve animals. They were housed in a polypen (thick polythene sheeting draped over and fixed to a steel frame). Immediately before, and after the five month period of housing, all ewes were grazing on a neighbouring paddock. Lambing was completed in the polypen.

Commercially available pelleted feedstuff, with Cu content varying from 20 mg up to 82 mg Cu/kg, was supplemented with Zn up to 352 mg Zn/kg; an attempt was made to achieve a dietary ratio, Cu : Zn of 1:4 throughout the period of housing. No deaths occurred during the period of housing.

II. Results and Discussion

The plasma Cu and Zn concentrations in Dorset ewes were more variable than in either Clun or Finn breeds, and also responded more rapidly to dietary changes in concentration of these metals. The immediate effect of housing was a significantly higher ($P<0.001$) mean plasma Cu concentration (Table 1. Day-0) in Dorset

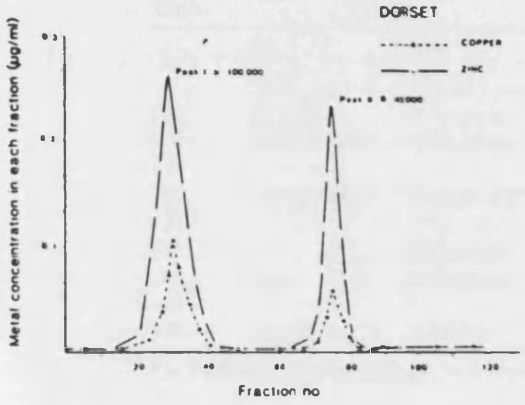

Fig 1

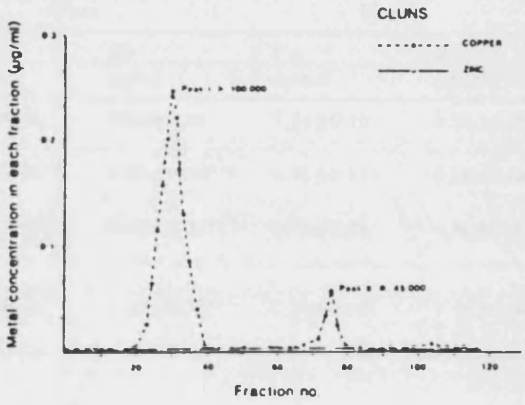

Fig 3

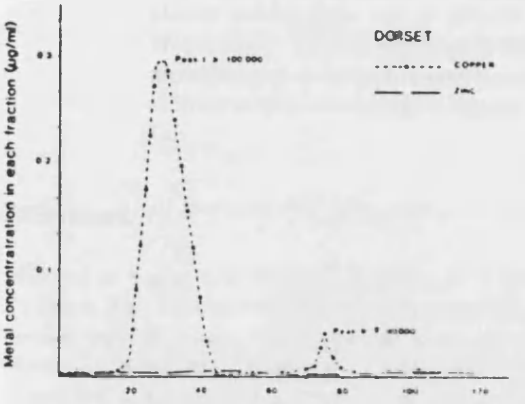

Fig 2

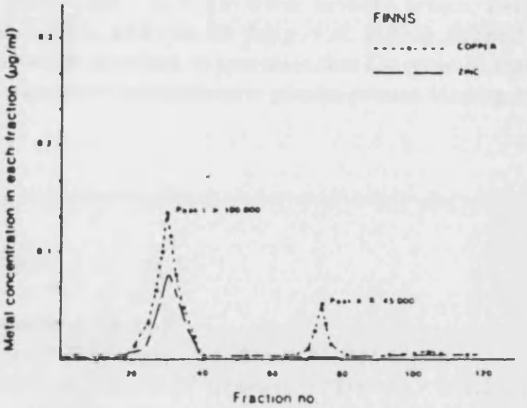

Fig 4

Fig. 1-4 *Elution profiles of Cu and Zn concentration in fractions of plasma protein separated on Sephadex G-75 column (5 x 90 cm equilibrated and eluted with 0.01 M Tris-HCl buffer, pH 8.6, containing 0.02% sodium azide.* 30 ml of plasma was applied on the column and a total of 120 fractions, 13.5 ml each, were collected at a flow rate of 60 ml/h. Protein bound Cu was found in two protein peaks : peak 1 of molecular weight > 100,000, and peak 2, approx. 45,000. Fig. 1 Dorset ewes on Day-0, first day in the polypen. Fig. 2 Dorset ewes after 21 days of housing. Figs. 3 and 4, Clun and Finn ewes respectively on Day-0.

and Clun ewes than before housing; in contrast this stress effect of housing was not observed in Finn ewes which showed no significant increase in plasma Cu.

Fractionation of plasma proteins from Dorset ewes showed that the bound Cu in Peak I representing a mean 0.061 g. atom Cu per mole after 21 d of housing (Fig. 2) was significantly higher ($P<0.01$) than the bound copper, mean 0.030g atom Cu per mole, in this peak at Day-0, Fig. 1. The bound copper at 21 d decreased very significantly ($P<0.001$) by 48 d. This indicated antagonism of Zn to Cu decreasing the transport of Cu to liver and thus showed the protective effect of a Zn-supplemented diet for intensive rearing of Dorset lambs threatened with toxicity.

The mean Cu bound to Peak I on Day-0 in Finn ewes, 0.033 g atom Cu per mole, was significantly lower ($P<0.001$) than mean bound Cu, 0.048g atom Cu per mole, in Clun ewes on Day-0 (Figs. 4 and 3 respectively). The pregnant Finn ewes were the least affected by the stress of housing on plasma bound Cu.

Table 1 Mean plasma Cu and Zn concentration in Dorset, Clun and Finn sheep

Days	Dorset		Clun		Finn	
	Cu µg/ml	Zn µg/ml	Cu µg/ml	Zn µg/ml	Cu µg/ml	Zn µg/ml
0	2.31±0.13	1.00±0.07	1.43±0.08	0.66±0.01	1.14±0.16	0.98±0.05
4	1.58±0.10***	1.00±0.06				
14			1.17±0.06**	0.86±0.03***	0.91±0.18	0.60±0.04***
21	1.29±0.07***	0.66±0.55***				
27			1.15±0.04**	0.84±0.03***	0.78±0.11	0.86±0.04
35	1.55±0.09***	0.82±0.05				
48	1.12±0.07***	0.72±0.03**				
88			1.43±0.08	1.10±0.02***	1.21±0.07	1.15±0.04*
105	1.62±0.07***	1.18±0.03				

* = $P<0.05$ ** = $P<0.01$ *** = $P<0.001$

The results suggest that the three breeds differed considerably with regard to the Cu bound to Peak I and thus the efficiency of Cu homeostasis, whilst depending on dietary ratios of Zn : Cu influencing the plasma protein (mol. wt. >100,000) binding capacity for Cu, might differ between breeds. Evidence by Wiener *et al.* (1978) and later Woolliams *et al.* (1982), and van der Berg *et al.* (1983), suggest that Cu metabolism may differ between breeds. It is reasonable therefore to speculate that Cu could be transported to livers in different breeds at speeds which are dependent on competitive plasma protein binding of Zn and Cu.

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INTERNATIONAL SYMPOSIUM ON MINERALS AND TRACE ELEMENTS IN HUMAN DISEASES

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Abstract 50

IN VITRO ZINC BINDING TO PLASMA ALPHA 2-MACROGLOBULIN IN SHEEP FED COPPER DEFICIENT AND COPPER EXCESS DIETS

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Zinc bound to alpha 2-macroglobulin (alpha 2-M) accounts for nearly 30 to 40 percent of the total plasma zinc. In this study Cu deficient diet (<1.0 mg Cu/kg and 50 mg Zn/kg diet) and Cu excess diet (250 mg Cu/kg and 200 mg Zn/kg diet) were fed to sheep for a period of 7 weeks. Blood samples were obtained and alpha 2-M from plasma was separated using immunoadsorbent chromatography. Zn binding to alpha 2-M was studied at two levels of zinc: level I, 150 µg Zn; level II, 1500 µg Zn. In Cu deficient sheep alpha 2-M incubated with both levels of Zn showed significantly ($P < 0.001$) increased Zn binding over the first 4 weeks. At Zn level II (1500 µg Zn), the increased Zn binding was maintained over the entire 6 weeks, whilst at Zn level I (150 µg Zn) this increased Zn binding to alpha 2-M was subsequently lost after an initial period of 4 weeks. Thus confirming the existence of different binding sites on alpha 2-M and highlighting the importance of the concentration of zinc in Cu deficient animals. These results also confirm the antagonisms between copper and zinc.